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Cell cycle progression and proliferation are mediated by the cyclin-dependent kinases (CDKs). These growth-promoting factors are counter-balanced by another family of inhibitory proteins that includes p21/WAF1, a regulator that is thought to restrain cell growth primarily as a consequence of its ability to inhibit CDKs. It is known that p21 assembles into multiple complexes with CDKs and the DNA polymerase processivity factor, PCNA, in vivo, but despite this knowledge, the reason for the existence of diverse complexes with p21 is not clear. In addition, the activity of p21-CDK complexes in vivo has been controversial, since there have been reports of p21-CDK complexes that retain kinase activity. Moreover, it is not known whether all components of p21 complexes have been identified. Given the role of p21 as a downstream target of the p53 checkpoint protein and the role of this protein and related ones in kinase inhibition and growth control, we seek to understand how p21 function is related to normal cell growth and whether loss of function results in transformation of human mammary cells.

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4) TABLE OF CONTENTS

PAGE	SECTION
1	Front Cover
2	SF 298 Report Documentation Page
3	Foreword
4	Table of Contents
5-7	Introduction
7-23	Body
23-24	Conclusions
24-26	References
	Appendix (including manuscripts in press)

5. INTRODUCTION

Background

Mammalian cell cycle progression is controlled on multiple levels. In part, growth is controlled by the periodic activation of specific cyclin-dependent kinases (cdks) at appropriate times. Cdk activation requires an association with a regulatory, or cyclin, subunit. Two cyclins, D and E, have been shown to function during G1 phase in specific combinations with cdk2, cdk4, and cdk6, and the activities of these proteins are necessary for S phase entry (reviewed in Sherr and Roberts, 1995). Cyclin A- and cyclin B-dependent kinases are activated subsequently, during and after S phase. The retinoblastoma (pRB) tumor suppressor protein, a negative regulator of the transcription factor E2F, is a well-established target of cyclin D/cdk4 and cyclin D/cdk6. E2F plays a pivotal role in cell growth because it is thought to activate genes necessary for S phase entry (reviewed in Dyson, 1998). Phosphorylation of pRB negates its growth suppressive properties by preventing its interaction with E2F and other transcription factors that promote entry into S phase.

Activity of the cyclins and cdks is counter-balanced by growth-suppressing inhibitors of these kinases. At least seven cdk inhibitors (CKIs) have been identified. These fall into two classes: the INK4 (inhibitors of cdk4) proteins (p15, p16, p18 and p19) inhibit cyclin D-dependent kinases specifically, while a second class, composed of p21 (also known as Cip1,WAF1, Sdi1, CAP20; henceforth, p21), p27, and p57, inhibits a wide range of kinases. CKIs appear to be components of multi-protein complexes in normal cycling cells. In normal diploid human cells, p21 has been found in quaternary complexes that contain proliferating cell nuclear antigen (PCNA) and cyclin/cdk pairs. Interestingly, oncogenic transformation promoted profound rearrangement of these complexes (Xiong et al., 1993). In transformed cells, cdk4 no longer associated with cyclin D/PCNA/p21 complexes and instead associated exclusively with the p16 protein. Likewise, cyclin B/cdc2 and cyclin A/cdk2 complexes with PCNA and p21 were disrupted. This pattern of rearrangement also occurred in cells transformed by other viral oncoproteins (encoded by adenovirus and papillomavirus), suggesting that cellular transformation might occur as a consequence of incorrect formation of cyclin/kinase/PCNA/CKI complexes. These data suggested that one pathway toward oncogenesis may involve alteration of the cell cycle machinery, although the exact biochemical consequences of this rearrangement are not known. Thus, detailed biochemical studies of the causes of these alterations may shed light on the mechanisms of transformation.

That CKIs play a pivotal role in cell cycle progression and oncogenic transformation has been well established by many experiments. First, each of these inhibitors can promote G1 phase arrest when overexpressed in certain tissue culture cell lines (reviewed in Sherr and Roberts, 1995). Second, transforming growth factor-β (TGF-β)-induced G1 arrest of certain cells may be explained by the induced synthesis of one INK4 family member, p15, implicating this CKI as an effector of cell cycle arrest (Hannon, 1994). Third, as previously mentioned, complexes with CKIsundergo rearrangements in transformed cells. Finally, p16 expression can suppress neoplastic transformation of fibroblasts by H-ras and c-myc (Serrano, 1995).

Biochemical experiments have indicated that p21 has a direct role in the inhibition of kinases promoting traversal of G1, including cdk2, cdk3, cdk4, and cdk6 (Harper et al., 1995). In addition to its kinase inhibitory activity, p21 may also block DNA replication. This was demonstrated using an SV40-based DNA replication system, in which p21 inhibited the ability of PCNA to stimulate the processivity of DNA polymerase δ (Flores-Rozas et al., 1994; Waga et al., 1994). DNA replication in *Xenopus* extracts was also blocked by incubation with the cdk-inhibitory domain of p21 (Chen et al., 1995). Previous studies, however, suggested that p21 can exist in active kinase complexes and that low levels of p21 enhanced cyclin/kinase complex formation; the kinase activity of these complexes could, however, be abolished by the further addition of p21, suggesting that kinase inhibition required the binding of two p21 molecules (Zhang et al., 1994; Harper et al., 1995; LaBaer et al., 1997). As described below, these studies have not addressed the specific activity of cellular p21/cyclin/CDK complexes, and definitive proof that such complexes are active is lacking. Although CKIs do show some binding preferences in vitro, the function of each of these individual complexes is currently unknown. Moreover, the reasons for the existence of so many inhibitors and whether there is redundancy of function is not clear. Redundancy may explain why mice lacking p21 are viable and are not prone to tumorigenesis or to developmental defects (Brugarolas et al., 1995; Deng et al., 1995; Brugarolas et al., 1998). Clearly, though, cells lacking p21 are not normal because the ability of p21-deficient cells to execute the p53-mediated G1 checkpoint is compromised.

Structure-function studies of p21 suggest that this protein has two separate domains (Goubin and Ducommun, 1995; Chen et al., 1995; Chen et al., 1996; Gulbis et al., 1996)(see Figure 1). An amino-terminal domain (residues 1-80) binds to and inhibits cyclin/CDK complexes, and a carboxy-terminal domain (residues 139-160) binds to PCNA and may suppress DNA replication. The former domain contains both cyclin-binding (residues 10-40) and CDK-binding (residues 40-60) regions and is highly homologous to the cognate region of p27, a closely related family member. A crystallographic study of a ternary complex containing cyclin A, cdk2, and an amino-terminal fragment of p27 suggested that a region of p21 between residues 74-79 (homologous to p27 residues 85-90) may be deeply buried in the catalytic cleft of CDK, occupying the ATP-binding site and playing a critical role in inhibition of CDK activity (Russo et al., 1996). In this study, dimerization of p27 was not necessary for kinase inhibition. Interestingly, this study also confirmed the structural importance of a cyclin-binding motif in p27 (the LFG or Cy amino acid motif) that is conserved in p21 and p57, as well as the pRB-related proteins p107 and p130 (Zhu et al., 1995). This domain in p107 is required for binding cdk2 complexes with cyclin A and cyclin E, inhibition of these associated kinases, and in some settings, growth suppression (Zhu et al., 1995).

Purpose of present work/Experimental approach

Despite a plethora of studies on p21, many questions remain unanswered. At the simplest level, our understanding of why there are so many complexes containing p21, and how they change during the cell cycle in normal and transformed cells is not yet complete. How does p21 binding alter kinase activity in vivo? Are there two states of p21-bound complexes in cells, i.e., kinase-active and inactive complexes? Structural studies of p27, the p21 monomer, and the p21-Cdk2 complex (Kriwacki et al., 1996) made use of recombinant proteins (or protein fragments) that were assembled into complexes in vitro.

Because of the limitations of reconstituting such complexes, we sought to determine the molecular architecture of p21 complexes in vivo to gain a better sense of how p21 can function as a kinase inhibitor. We have also continued to ask whether all proteins associated with p21 have been identified and whether p21 regulates E2F complexes. Thus, we have extended our previous biochemical studies with the hope of answering each of these important questions. Effective therapeutic strategies for the treatment of mammary and other cancers might be envisioned when molecular details such as these are fully understood.

In the past year, we have pursued the following specific goals:

1) To identify and characterize p21-containing complexes in normal and transformed human cells, including multiple breast carcinoma cell lines, during various stages of the cell cycle. The subcellular distribution of such complexes in normal fibroblasts was determined during cell proliferation.

2) To characterize novel cell cycle-regulated proteins associated with p21.

3) To identify transcription factors of the E2F family that associate with p21 and study the regulatory properties of such complexes. Simultaneously, we have studied the regulation of cdk activity by the pRB-related protein p107, which bears some structural and functional homology to p21.

6. BODY

Substantial progress has been made in several of the areas listed above, and some of these novel findings, described below, have suggested that we concentrate on certain areas and put less effort into others. In addition, a slight adjustment to our original Statement of Work has been made to compensate for the recent progress on p21 by other research groups as described below.

Some of our findings have been submitted as manuscripts for publication and are now in press. Copies of these two manuscripts (Castano et al., *Mol. Cell Biol.* and Cai and Dynlacht, *Proc. Natl. Acad. Sci.*) can be found in the Appendix section.

1) **Characterization of known p21-containing complexes in normal and transformed human breast cells**

A) precise epitope mapping of antibodies raised against p21
B) activity of CDKs in complexes with p21
C) abundance and sub-cellular localization of p21 during the cell cycle
D) existence of "free" p21 in normal and transformed cells during the cell cycle

It has been proposed that active kinase complexes containing p21 may exist in the cell, raising a question as to why a kinase inhibitor would associate with active kinases without altering their activities. This paradox was believed to be resolved by in vitro experiments that suggested the stoichiometry of p21 relative to cyclin/CDK components could alter kinase activity: Thus, in the presence of low levels of p21, kinases were active, but elevated

levels of p21 extinguished their activity (Zhang et al., 1994; Harper et al., 1995). We sought to resolve this paradox by studying the activity of endogenous p21-containing complexes. We also sought to address the broader question of how the relative levels of p21, cyclins, CDKs, and PCNA in multimeric complexes correspond to total cyclin-dependent kinase activity, and how differences in such levels relate to cell cycle progression in normal human fibroblasts. Once these questions had been addressed with normal cells, we would then focus on cancer cells, specifically, breast carcinoma cells. Our recent experiments concerning regulation of CDK activity by p21 in vivo are described below.

A) Epitope mapping of p21 MAbs

The first step toward addressing the above questions entailed the fine mapping of epitopes recognized by each of our p21 MAbs. Although the epitopes had been mapped less precisely previously (Dynlacht et al., 1997), we sought to get structural details of p21 complexes as well as to confirm that each of the antibodies mapped to unique epitopes. We accomplished this using a peptide array of 13-mers spanning p21 that differed sequentially by two carboxy-terminal residues. Using this method, we distinguished at least six different monoclonal antibodies recognizing several epitopes corresponding to known functional domains (Figure 1).

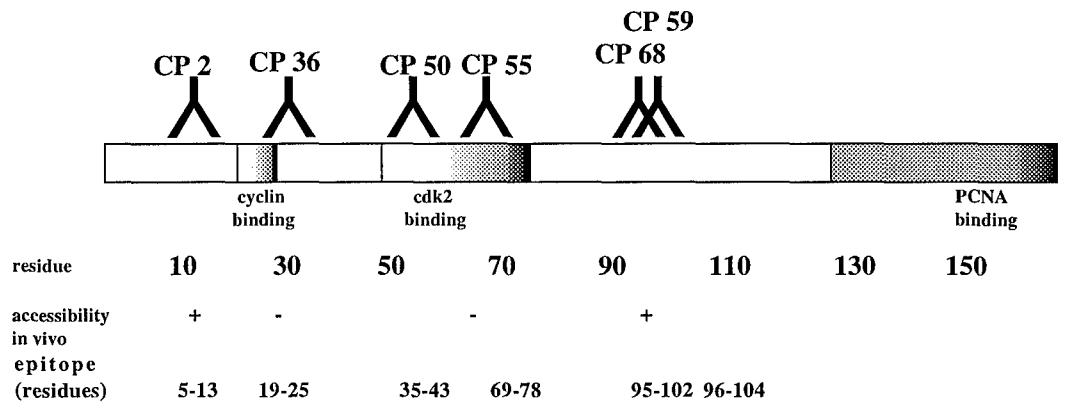


Figure 1. Fine mapping of epitopes recognized by anti-p21 monoclonal antibodies. Each p21 Mab was mapped using a peptide array. The residues recognized by each are indicated at the bottom, and important functional domains are also shown. The accessibility of each epitope in native cell extracts is also depicted.

In addition to these antibodies, we also made use of a commercially available one (C-19; Santa Cruz Biotech.) that recognized an epitope that precisedly overlapped with the PCNA-binding domain (residues 144-164). In agreement with our previous studies (Dynlacht et al., 1997), we demonstrated that certain antibodies (CP2, CP59, and CP68) immunoprecipitated all known cellular complexes containing cyclins, Cdks, PCNA, and p21, while another one (CP36) immunoprecipitated only cyclin A/cdk2/p21, and a third group (CP55) immunoprecipitated only "free" p21 devoid of cyclins and Cdks (although trace amounts of PCNA and Cdk4 were sometimes detected in the latter immunoprecipitates). These findings are shown in Figure 2.

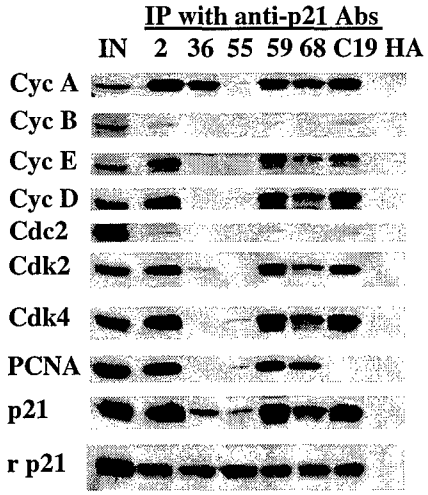


Figure 2. Recognition of distinct p21 complexes by Mabs is determined by masking of epitopes in p21 by associated proteins. Extracts of normal human fibroblasts were immunoprecipitated with the antibodies indicated at the top of the figure and subjected to western blotting with the antibodies indicated on the left. For comparison, precipitations of purified, recombinant (bacterially expressed) p21 are shown in the bottom-most panel.

Interestingly, the differences observed here were not due to variations in the affinity of each antibody for p21 itself, since each of the antibodies immunoprecipitated equivalent amounts of the recombinant protein. Instead, the inability of some to recognize p21 and associated proteins resulted from the masking of epitopes in the endogenous complexes. This was confirmed by performing immunoprecipitations of native cell extracts and extracts denatured with urea (not shown). There was a very strong correlation between the ability of a given antibody to recognize a specific complex and the epitope to which it mapped. For example, CP36 recognized a region that corresponds exactly with the LFG/Cy cyclin-binding motif conserved in the p21/p27/p57 family as well as the pRB-related proteins p107 and p130. Cyclin binding to this motif would be predicted to prevent accessibility to CP36, and this is exactly the case. Apparently, cyclin A (but not cyclins B, D, or E) can also bind to p21 in vivo through a second, recently identified, carboxy-terminal Cy motif (Chen et al., 1996). In addition, residues 69-78 are inaccessible in vivo since CP55, which recognizes this epitope, is able to precipitate only "free" p21 (see below). This region corresponds to a 3_{10} helix in p27 that is buried deeply in the catalytic cleft of cdk2 and may interfere with ATP binding (Russo et al., 1996). Our experiments for the first time begin to elucidate the architecture of p21 complexes in vivo.

B) Activity of p21 complexes during cell cycle progression

As documented in our previous Annual Report, immunoprecipitation of ^{35}S-methionine-labeled cell extracts yielded highly enriched p21 complexes that contained each of the expected associated proteins and few contaminating proteins upon fluorographic exposure. One important objective in our original Statement of Work was to determine the kinase activity of endogenous complexes containing p21. Because we had carefully characterized each p21 MAb, and because immunoprecipitates of crude extracts were nearly homogeneous, we were confident that we could directly measure any specific kinase

activity that derived from native complexes without detecting potential contaminating kinase activities.

When we performed these experiments using extracts from normal fibroblasts, we obtained the data in Figure 3.

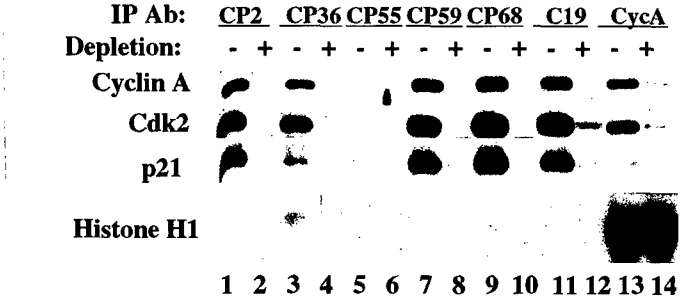


Figure 3. Immune precipitates of p21 MAb. Direct comparison of levels of associated cyclin A, cdk2, and p21 and the histone H1 kinase activity associated with each complex.

Clearly, none of the immunoprecipitates contained significant amounts of kinase activity when compared against cyclin A immunoprecipitates. Weak histone H1 phosphorylation was detected in CP36 immunoprecipitates. However, it was important to calculate the specific activity of each immunoprecipitated complex, and we did so by performing densitometry on the immunoblots (using NIH Image software) and by using the PhosphorImager (PI) to quantitate kinase activity. We used the formula: specific activity= PI units (arbitrary)/cdk2 densitometry units (arbitrary). Using this method, we found that cyclin A/cdk2 complexes lacking p21 were greater than 100-fold more active than the corresponding ones containing p21. Also noteworthy was the fact that immunoprecipitation of cyclin A and cdk2 complexes after depletion of p21 complexes did not result in reduced kinase activity relative to non-depeleted controls. The ability of our MAbs to completely extracts of p21 has been used to great advantage in our cell cycle progression experiments (see below). These results challenge the notion that cellular p21/cyclin A/cdk2 complexes have kinase activity.

The above experiments were performed using asynchronous WI38 extracts. We considered the possibility that p21 complexes with cyclin-dependent kinases might display activity at specific stages of the cell cycle and that a survey of an asynchronous population would preclude a measurement of such activity. To rule this out, we synchronized WI38 cells by two methods, serum deprivation and contact inhibition. Both methods resulted in the arrest of a majority of cells (greater than 85%) as determined by FACS analysis (see Fig. 4; data shown for serum deprivation/re-stimulation only). The cells re-entered the cell cycle upon serum addition, entering S phase between 16 and 20 hours after re-stimulation. The largest fraction of cells were in S phase 24 hours after re-stimulation. Extracts of samples collected at each time point during serum deprivation and re-stimulation were subjected to direct western blotting to determine the total amounts of cyclin A, cdk2, and p21 present during cell cycle re-entry.

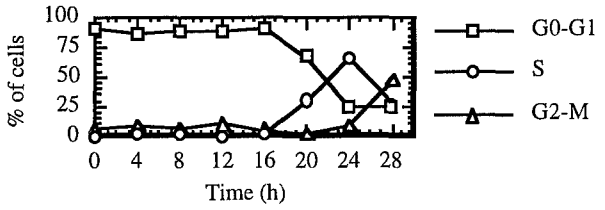
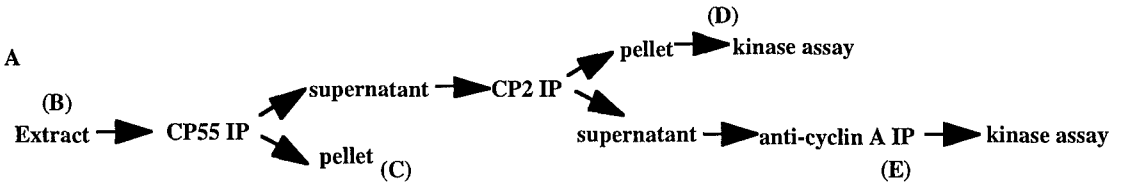


Figure 4. Cell cycle synchronization of normal human fibroblasts using serum deprivation and re-stimulation. Time after serum addition and percentage of cells in each phase of the cell cycle are shown.

As expected from previous studies, the abundance of p21 protein did not fluctuate dramatically during this time course, although p21 levels did increase by approximately two-fold upon serum stimulation (compare 0 and 4 h time points). In contrast, cyclin A levels rose sharply as cells entered S phase, peaking between 20-24 hours post-stimulation. To estimate the level of cyclin A/cdk2 kinase activity associated with p21 as cells exited G0 and progressed through G1 and S phases, we used the strategy shown in Figure 5A.



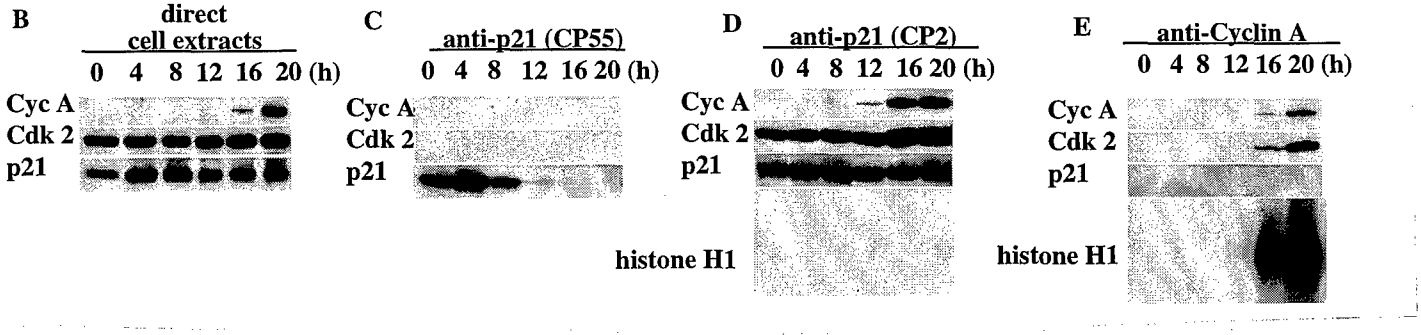


Figure 5A-E. Determining the kinase activity associated with p21 during cell cycle progression. Immunoprecipitations, western blotting, and kinase assays were performed as described in part A. Parts B-E show that as cells enter S phase, cyclin A/cdk2 abundance exceeds that of p21 resulting in robust kinase activity. Interestingly, the use of CP55 in part C shows the existence of abundant "free" p21 during early G1.

Western blotting was performed at each stage to estimate the levels of each protein of interest. Since the ultimate goal of these experiments was to determine the kinase activity of cyclin A/cdk2/p21 complexes and cyclin A/cdk2 that was completely devoid of p21, we initially performed immunoprecipitations on extracts from each time point with CP55 to inspect "free" p21 levels and to remove this factor from further activity considerations. As

expected from our initial studies, CP55 immunoprecipitated p21 to the exclusion of both cyclin A and cdk2 (Figure 5C). Remarkably, we observed a vast excess of "free" p21 in quiescent and early G1 phase cells. Between 8 and 12 hours post-stimulation, however, "free" p21 levels dropped precipitously, although the total amount of p21 during this time remained largely unchanged (compare 4-12 hour time points in panels C and D). Furthermore, this cell cycle-dependent immunoprecipitation of p21 by CP55 indicates that this antibody is not simply disrupting p21 complexes and thereby artificially creating "free" p21.

After depletion of "free" p21 from extracts at each time point with CP55, we subsequently immunoprecipitated the same extracts with CP2, which recognizes all known p21-associated cyclin/Cdk complexes. Although robust amounts of p21 and cdk2 were precipitated throughout the time course, cyclin A was not detected until approximately 12 hours after serum addition, as expected from western blots of total cell lysate. However, association between this newly synthesized cyclin A/cdk2 and p21 resulted in negligible levels of histone H1 kinase activity. From these data we conclude that inhibition of cdk2 complexes through association with p21 in vivo is independent of cell cycle position.

The use of depleted extracts allowed us to estimate the abundance and kinase activity of cyclin A/Cdk2 complexes lacking p21 as cells traversed the G1 phase. Immunoprecipitation of such extracts with anti-cyclin A antibodies revealed the presence of cyclin A/Cdk2 16 and 20 hours after serum stimulation, and as expected, this kinase was not associated with p21. This indicated that most of the cyclin A/cdk2 is associated with p21 in these cells at this point in the cell cycle. Interestingly, at 16 and 20 hours post-stimulation, there was considerably less cyclin A and Cdk2 in binary complexes as compared to the amount present in the ternary complex with p21 obtained by immunoprecipitation with CP2, although the amount of kinase activity in the former complex was much greater. Indeed, when we calculated the specific Cdk2 activity of both complexes (as described above), we found that the activity of the cyclin A/Cdk2 complex was 166-fold and 158-fold higher than the p21/cyclin A/cdk2 complex at 16 and 20 hours post-stimulation, respectively.

We also induced quiescence in WI38 cells by a second method, namely, contact inhibition, to alleviate the possibility that levels of p21 were artificially induced by serum stimulation of quiescent cells. This treatment resulted in growth arrest of a substantial fraction of cells (greater than 80%), and high levels of "free" p21 similar to those described above were observed exclusively at early time points after release from contact inhibition (not shown). Taken together with the data in Figure 5, we speculate that the existence of "free" p21 may be an intrinsic feature of early G1 cells, although confirmation will require the testing of additional cell lines (see below).

We draw an important conclusion from these data. Namely, significant levels of histone H1 kinase activity are not associated with p21 during cell cycle progression from G0 to S phase, when cyclin A/cdk2 and cyclin A/cdk2/p21 complexes are compared. Having made these observations in normal fibroblasts, we intend to expand this line of research and are currently investigating p21-associated kinase activity in a large number of breast carcinoma cell lines, using normal breast cell lines for comparision. These future experiments are detailed further in section C below.

C) Existence of "free" p21 in normal and transformed cells during cell cycle progression

From the data described above, it is clear that one p21 MAb, CP55, was unable to recognize known complexes of p21 in cell extracts, since the epitope recognized by this antibody maps between residues 69 and 78, and crystallographic studies of p27 suggest that an analogous region in p21 forms a 3_{10} helix that may be buried deeply in the cdk2 protein (Russo et al., 1996).

To rule out the possibility that CP55 immunoprecipitated a complex of other proteins not detected in our western blotting analysis, we estimated the size of p21 complexes in early G1 (4 h after serum restimulation) and S phases (24 h after serum stimulation) by subjecting extracts of WI38 cells to S-300 gel filtration chromatography (Fig. 6A). Extracts from early G1 cells contained both "free" p21, which migrated at a position (between the 12 kDa and 29 kDa markers) predicted for a monomer, and larger complexes (between 67 kDa and 443 kDa markers) that contained p21 in association with cyclin D/Cdk4, cyclin E/Cdk2, and PCNA. Of these two pools, CP55 was solely capable of immunoprecipitating the low molecular fraction between 12 and 29 kDa in size. The p21 fraction immunoprecipitated by CP55 co-migrates exactly with recombinant p21 purified from bacteria (data not shown), suggesting that a form of p21 free of interacting proteins was recognized by this antibody. In striking contrast, p21 is largely confined to high molecular weight complexes in extracts of S phase cells.

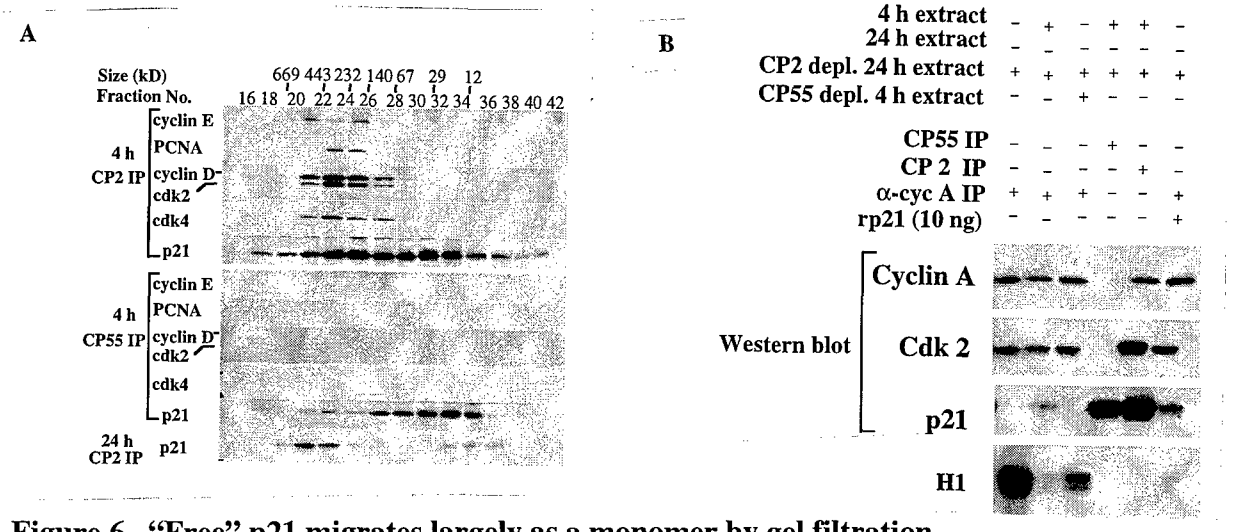


Figure 6. "Free" p21 migrates largely as a monomer by gel filtration chromatography. (A) An S-300 column was used to separate p21 complexes in WI38 extracts. 500 µg of protein derived from cells 4 h after serum stimulation were loaded on the column. Fractions were collected, halved, and immunoprecipitated with either CP2 or CP55 as labeled. The samples were analyzed by western blotting to reveal p21 and its complexes. (B) 4 h extracts can inhibit cyclinA/Cdk2 in S phase. For lanes 2-5, samples were mixed and incubated before immunoprecipitation. The bottom-most panel shows the histone H1 kinase activity in each reaction. Lane 6 is a control in which cyclin A/Cdk2 was incubated with 10 ng of purified, recombinant p21.

We tested whether mixing extracts from cells stimulated for 4h would inhibit cyclin A/Cdk2 kinase activity found in 24 h cell extracts, and this was indeed the case (Figure 6B,

compare lanes 1 and 2). Depletion of "free" p21 from identical 4h extracts with CP55 partially reversed its kinase inhibitory activity, suggesting that this CKI was at least partly responsible for the inhibition of kinase activity in early G1 cells.

We draw several important conclusions from these data. First, although total p21 levels do not change dramatically during the cell cycle, the amount of "free" p21 (defined as that population of p21 that is not associated with cyclin/cdk complexes) is high in early G1 and drops sharply as cells progress toward S phase. We speculate that excess p21 ensures that cyclin/cdk complexes are inactive in early G1. As cells progress through G1, cyclin A levels rise, and the levels of cyclin A/cdk2 surpass those of p21, providing the impetus for the onset of S phase. Thus, our data support a threshold hypothesis for p21, which had been proposed based on in vitro experiments (Harper et al., 1995), in which the ratio of cdks to p21 determines enzymatic activity.

To test the generality of this hypothesis and perhaps extend our conclusions to other normal and transformed cells, we have recently included both normal and cancerous mammary cell lines. Preliminary data are shown in Figure 7.

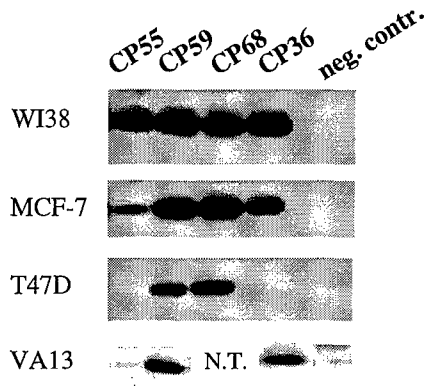


Figure 7. Reduction or absence of "free" p21 in several transformed cell lines. Equivalent amounts of total cell protein was immunoprecipitated with the indicated antibodies and immuno-blotted with CP36 (anti-p21).

Interestingly, both WI38-VA13 (an oncogenically transformed version of WI38) and T47D, two cancer cell lines, have no detectable "free" p21, while another breast cancer cell line, MCF-7, has much reduced levels when compared with WI38 (normal) cells. Furthermore, CP36 immunoprecipitated nearly undetectable amounts of p21 from T47D extracts, suggesting that these breast cancer cells may exhibit a different constellation of p21 complexes than normal cells. This idea is currently under investigation.

We have encountered some difficulties in furthering these experiments. For example, many mammary carcinoma cell lines that we have tested (MDA-MB-231, MDA-MB-468, Hs578T) contain very little or no p21 due to p53 mutation (not shown). Thus, cell lines lacking p21 will not be considered further, while experiments with cell lines containing reduced amounts must be normalized to other cell lines for total amounts of p21. In addition, in contemplating cell cycle synchronization experiments, we have found that certain breast cancer cell lines (MCF-7 and T47D) were not readily arrested by serum deprivation. Such protocols will be attempted again, and if this method continues to be

unsuccessful, we will attempt to synchronize these cells with drug treatments (such as nocodazole or colcemid) followed by removal of the drug.

Thus, we will need to survey several additional cell lines before reaching conclusions about a general mechanism involving p21 as a titratable inhibitor whose levels determine S phase onset. However, this model makes several testable predictions. For example, cell lines that do not have an excess of p21 relative to cyclin A/cdk2 may have considerably elevated levels of cyclin A-associated histone H1 kinase activity and perhaps a shortened G1 phase. If these cells compensate for loss of p21 by up-regulating other CKIs, however, a difference in kinase activity and cell cycle kinetics may not be observed.

D) Abundance and sub-cellular localization of p21 during the cell cycle

We have put forth some effort into studying the sub-cellular localization of p21 complexes. In these experiments, we have consistently reproduced our initial biochemical findings that the p21 protein can be found in both nuclear and cytosolic fractions in both normal fibroblasts and breast carcinoma cell lines.

We were therefore most interested to determine the localization of endogenous p21, initially using WI38 cells. To this end, we first tested dilutions (1:100) of each anti-p21 antibody in immunoblotting experiments. CP36 detected primarily p21 with little background, and this antibody was chosen for subsequent immunofluorescence experiments (data not shown). When we performed immuno-localization experiments with normal human (WI38) fibroblasts and CP36 or several other monoclonal antibodies, however, only nuclear staining was observed (Figure 8). Whether this is due to differences between the two assays (i.e., sensitivity of detection, native versus denatured state of antigen, etc.) or to our sub-cellular fractionation technique is not presently clear.

Figure 8 also shows the decline in nuclear p21 staining in conjunction with increased cyclin A staining as cells traversed S phase (compare 4 and 24 hour time points). Thus, 4 hours after serum stimulation, nearly all cells have abundant amounts of nuclear p21 (green), and no cyclin A (red) is visible. However, by 24 hours post-stimulation, fewer cells had strong p21 staining, many were positive for cyclin A, and almost none were positive for both cyclin A and p21 (yellow staining). Double staining with bromo-deoxyuridine (BrdU) revealed that each of the BrdU-positive cells was also positive for cyclin A but not for p21 (not shown). This result is consistent with our immunoprecipitation/western blotting experiments (see above) that suggest that as WI38 cells approach S phase, the abundance of cyclin A exceeds that of p21. This difference leads to robust cyclin A/cdk2 kinase activity and may help propel cells into S phase.

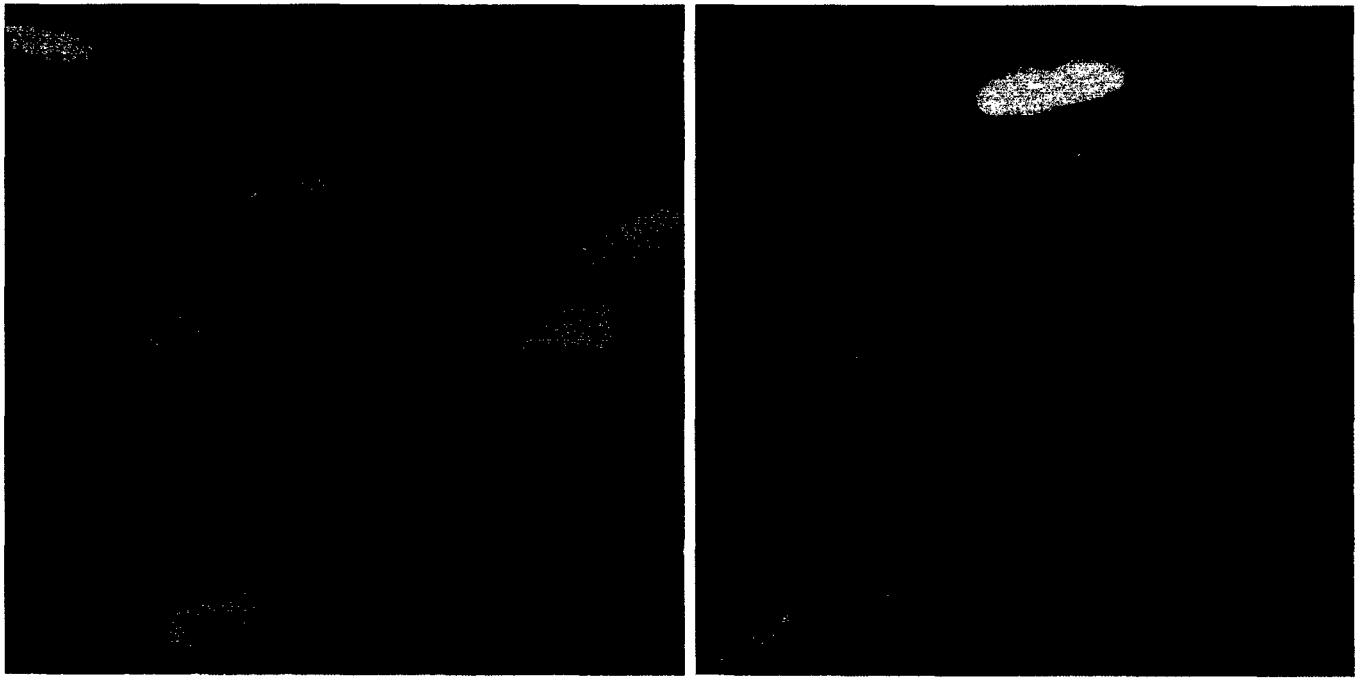


Figure 8. Immunolocalization of p21 and cyclin A. WI38 cells serum-starved and re-stimulated for the indicated lengths of time were incubated with antibodies against p21 and cyclin A. p21 (green) and cyclin A (red) immunofluorescence were observed by fluorescence microscopy.

While this work was in progress, Reed and colleagues published immuno-localization data that significantly overlapped with, and confirmed, these data (Dulic et al., 1998). Hence, we have not pursued these experiments with normal fibroblasts further. However, it will be interesting to test the localization of p21 in various transformed and mammary cancer cell lines, including VA13 and T47D which, as described in this Report, appear to have decreased levels of p21 in general and no "free" p21 (see below).

We have also pursued one other potentially interesting finding described in last year's annual Report. Namely, we have observed a CP36 cross-reactive band on immunoblots that migrated with slightly reduced mobility relative to recombinant and cellular p21. This protein was found exclusively in nuclear extracts of normal cells. Whether this represents a post-translational modification of p21, a related protein, or is simply a cross-reactive species was not clear. To this end, we attempted to immunoprecipitate this protein with our panel of p21 MAbs with the hope of treating the immunoprecipitates with protein phosphatases. However, we were completely unsuccessful in our attempts to precipitate this species with any MAb, including CP36 (data not shown). Thus, while the latter antibody is capable of recognizing this species in denatured form on western blots, we have not been able to immuno-purify it for further study. It is possible that some type of post-translational modification prevents recognition of this protein. Therefore, some effort could be made to isolate this protein after phosphatase treatment and/or denaturation of cell extracts. However, if we are unsuccessful at isolating this protein, no further experiments will be carried out to determine its identity.

2) Identifying novel p21-associated polypeptides

Previously we demonstrated that a few of our p21 MAbs immunoprecipitated several polypeptides (from metabolically labeled cells) in addition to the full complement of previously characterized polypeptides. Parallel immunoprecipitations ruled out known p21-related and associated proteins. We have performed additional experiments to further characterize p28 and p40, two potentially novel proteins that co-fractionated with p21 on glycerol gradients, sizing, and ion exhange columns. In an effort to identify polypeptides of the p21 complex that might interact in vivo with these putative novel proteins, we have carried out binding experiments with metabolically-labeled extracts of WI38 cells and glutathione-S-transferase (GST)- tagged full-length p21 as well as with amino- and carboxy-terminal fragments of p21. Neither p28 nor p40 was captured in preliminary binding experiments, suggesting that these proteins do not directly bind to p21 (data not shown). However, abundant amounts of labeled PCNA bound to the carboxy-terminal fragment of p21, confirming the direct nature of this interaction.

Our studies continue to be hampered by the extremely low abundance of such complexes in the cell lines we tested previously, including WI-38 and MCF-7. Small-scale pilot experiments aimed at immuno-purifying putative novel p21-associated proteins failed to produce silver-stainable quantities of protein (data not shown). We are continuing our search for optimal cell lines. One major problem is the lack of suspension cells that contain p21, since such cells would be useful for obtaining large quantities of starting material.

3) Regulation of E2F family members associated with p21 and p107/p130

We have made substantial progress in this area of the research proposal, and some of this work has resulted in a publication (see Appendix).

A) p21-E2F complex

First, we could unambiguously show, using specific antibodies, that the p21-associated E2F consisted of E2F-4 and DP-1. Interestingly, these E2F family members have been shown to associate with the p107 and p130 proteins as well (see below). Interestingly, we could also show that the p21-E2F interaction has been conserved in the mouse as well, since mouse embryo fibroblasts (MEFs) contained readily detectable p21-E2F complexes. Importantly, we could demonstrate that no E2F activity was immunoprecipitated with p21 MAbs from p21 knockout MEFs (a gift of T. Jacks). We could also detect E2F-4 polypeptides on immunoblots of anti-p21 immunoprecipitates (not shown). Our experiments suggesting the conservation of this interaction in another species have further convinced us of the necessity of further characterizing this complex.

Thus, we are attempting to characterize all components of the p21-E2F complex. Previously, we described a method using ion exchange (Q-sepharose) chromatography to separate complexes containing p21/cyclin A/cdk2 and p21/cyclin E/cdk2 from those containing p21/cyclin D/cdk4/PCNA. Initial E2F gel mobility shift assays of such Q-sepharose fractions suggested that the p21-E2F complexes co-purified with the latter complexes (data not shown). We are currently repeating these experiments, and if they are successful, we will attempt to purify the intact complex by immuno-affinity or DNA-affinity chromatography (with E2F binding site oligonucleotides covalently linked to sepharose). Once the components of this complex have been identified, we will attempt to reconstitute the complex in vitro and test the effects on E2F activation of transcription using highly purified factors (see for example Dynlacht et al., 1994).

B) The role of p107/p130 as p21-like kinase inhibitors

The pRB-related proteins p107 and p130 are thought to suppress growth in part through their associations with two important cell cycle kinases, cyclin A/cdk2 and cyclin E/cdk2, and the transcription factor E2F. Although each protein plays a critical role in cell proliferation, the functional consequences resulting from the association between growth suppressor, CDK, and transcription factor, have remained elusive. In an attempt to understand the properties of such complexes, we have studied the effect of p107 on the association with cyclin/cdk2 complexes, hoping that an understanding of contributions of p107 and p21 to overall cdk kinase activity could help us more fully understand the regulation of these kinases and how they go awry in normal and transformed cells. Loss of p107 or p130 has not been associated with human cancers, although a recent study indicated that the p130 gene was lost in a small cell lung carcinoma (Helin et al., 1997). It is also important to point out that the phenotype caused by loss of pRB-related proteins in mice is dramatically dependent on genetic background, and therefore, loss of these proteins contributed to tumorigenesis in some situations (Dyson, 1998). It remains to be shown whether any of these proteins are lost in breast tumors.

We had found previously that recombinant cyclin/cdk2 complexes with p107 or p130 were inactive (Woo et al., 1997). We therefore sought to determine first whether p107 and p130 were physiologically relevant CKIs, and we did so using two methods. First, we tested

cdk2-associated histone H1 kinase activity in MEFs that were either wild-type, p107-/-, or p107-/-; p130-/- (a gift of N. Dyson, E. Harlow, and T. Jacks). We noted a two-fold increase in kinase activity in cells that were deficient for p107 or both p107/p130, in agreement with a role for these proteins as cdk2 inhibitors (not shown). Although not dramatic, the presence of redundant CKIs in MEFs could account for this small difference. Indeed, p21-/- MEFs also display about a 2-4-fold increase in overall cdk2-associated kinase activity (Brugarolas et al., 1998). We also demonstrated the physiological effect of p107 a second way, by inducibly expressing low levels of ectopic p107. As shown in Figure 9, p107 was a potent kinase inhibitor under physiological conditions.

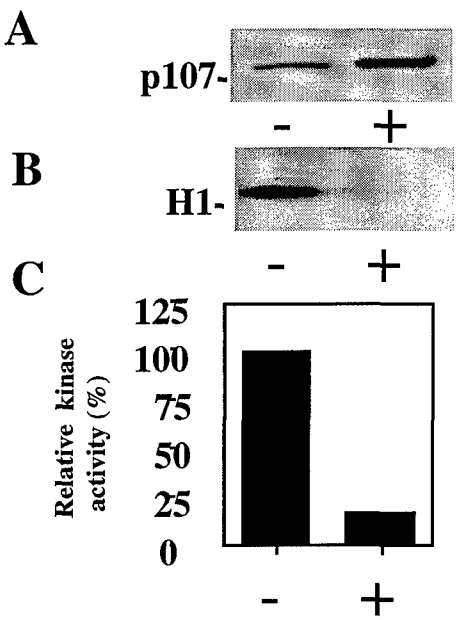


Figure 9. Induction of p107 using a tetracycline-repressible system causes a descrease in histone H1 kinase activity. Western blot of p107 is shown together with an H1 kinase assay and quantitation. Induction of p107 is indicated (+).

These data compelled us to perform kinetics experiments to address the inhibitory constant (K_i) of p107 and to compare it with p21. Interestingly, in kinase assays with recombinant, purified cyclin A/cdk2, p21 and p107 had almost indistinguishable K_is of approximately 1 nM (Figure 10).

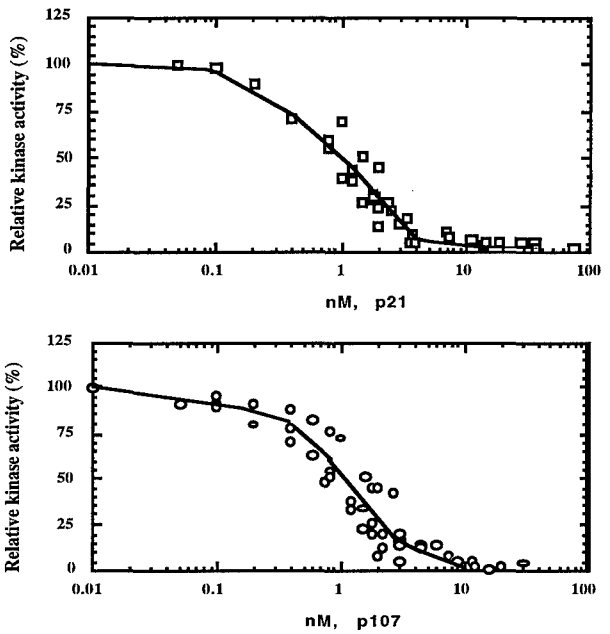


Figure 10. p21 and p107 display similar inhibitory constants toward cyclin A/cdk2. Histone H1 kinase assays were performed with recombinant, purified cyclin A/cdk2 (obtained from baculovirus-infected insect cells), p21 (bacterial), and p107 (insect cell). Phosphorylation was measured using a PhosphorImager.

There were, however, some differences between p21 and p107. For example, whereas the K_is of the two were similar for cyclin A/cdk2, p107 displayed a lower K_i (3.3 nM) toward cyclin E/cdk2 than p21 (14 nM)(not shown). Also, whereas p21 has been shown to be an effective inhibitor of cyclin D complexes (Harper et al., 1995), p107 was comparatively poor at inhibiting the same kinase (Figure 11). Here, amounts of p107 that abolished cyclin A/cdk2 activity only produced a 10% reduction in cyclin D/cdk4 activity toward a pRB substrate.

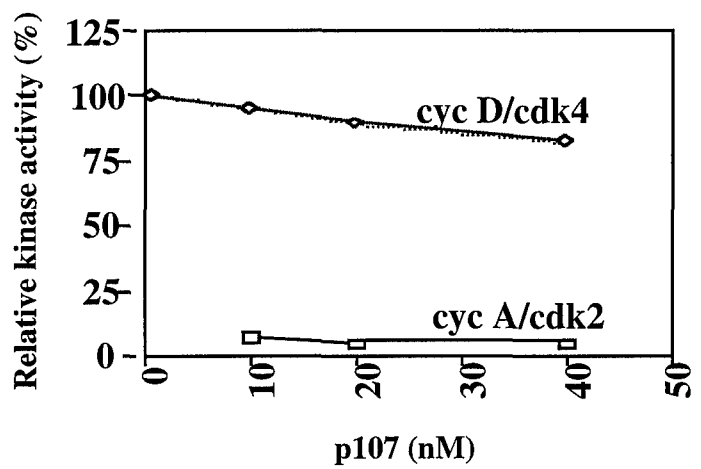


Figure 11. p107 displays an inhibitory preference toward cdk2 complexes. Using recombinant pRB as a substrate and purified kinases (obtained after expression in, and

purification from, insect cells), kinase reactions were performed, and the phosphorylation of pRB quantitated as in Figure 10.

Thus, the inhibitory constants for p107 are in the physiological range and further suggest the relevance of this protein toward cdk2 regulation in vivo. Moreover, the inhibitory spectra of p107 and p21 overlap, but they are not identical. Such differences could result in the inhibition of kinases at different stages of the cell cycle or at different times during development.

Since p107 is highly related to the retinoblastoma tumor suppressor protein, pRB, we tested the possibility that pRB might also inhibit cdks in a manner similar to p107. Interestingly, we found that pRB was completely unable to inhibit cyclin A/cdk2 and cyclin E/cdk2 (Figure 12 and not shown). Our results for the first time suggest that p107 and pRB can be distinguished biochemically by the unique ability of p107 to inhibit cdks. Thus, in addition to inhibiting E2F activity, p107 is capable of growth suppression by virtue of its ability to inhibit G1/S phase cyclin-dependent kinases. pRB does not use this mechanism to restrain proliferation.

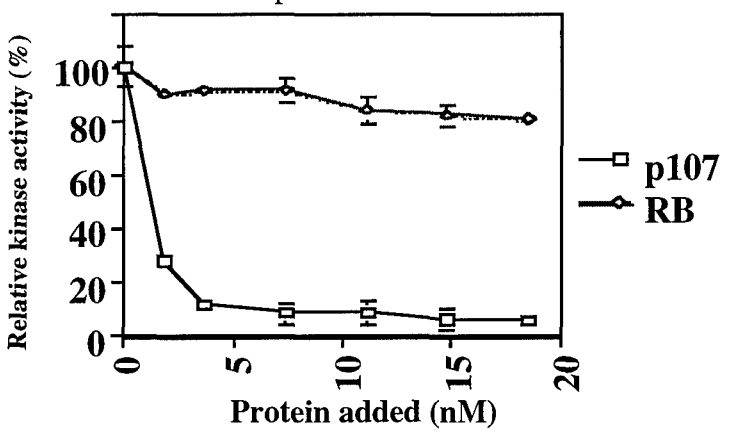
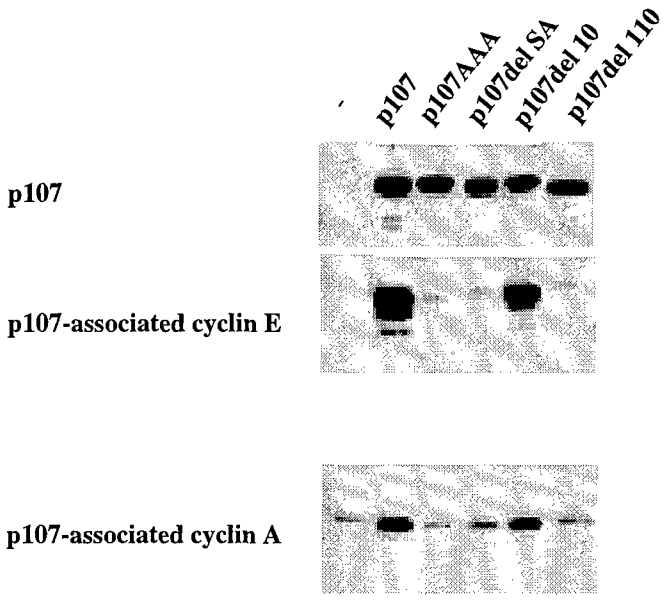


Figure 12. pRB is not an inhibitor of cyclin A/cdk2. The effect of increasing amounts of either pRB or p107 was tested on cyclin A/cdk2 phosphorylation of histone H1. Results were quantitated as in Figure 10.

We had shown in earlier experiments that an amino-terminal fragment of p107 was necessary for such inhibition of cdks (Woo et al., 1997). Visual inspection of this region of p107 revealed a sequence related to the LFG/Cy motif found in the "spacer" region of the p107 and p130 "pocket" domains and in p21/p27/p57 (Adams et al., 1996; Zhu et al., 1995; Chen et al., 1996). To determine whether this sequence was of functional importance in vivo, we performed deletion and point mutagenesis. We found that this region was critical for high affinity interactions with cyclins A and E in vivo and in vitro (Figure 13A and not shown). In addition, when we tested these mutants in growth suppression assays in the osteosarcoma Saos-2 and cervical carcinoma C33a cell lines, we found that loss of cyclin-binding resulted in near-complete reduction in growth suppressive activity as measured by FACS analysis of propidium iodide stained cells (Figure 13B).

A



B

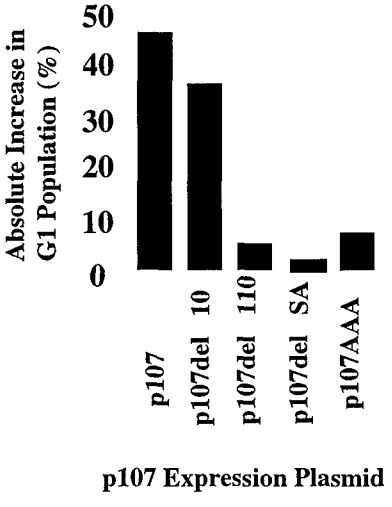


Figure 13. In vivo cyclin binding and growth suppression by p107 mutants. (A) Several deletion and point mutants were made for p107. Del 10 and 110 remove 10 and 110 amino-terminal residues, respectively, while del SA is a small amino-terminal deletion that removes the putative LFG/Cy motif. p107AAA is a triple point mutant that changes the C, R, and K residues (Cy motif amino acids 67-69) to A. Each of the p107 derivatives were expressed to equivalent levels in C33A cells, and p107 was immunoprecipitated. Western blots of immunoprecipitates were probed for cyclin E and cyclin A. (B) The growth suppressive effect of each construct was tested. Results are shown as an absolute increase in the percentage of G1 phase cells as determined by FACS analysis.

Thus, another striking similarity between p107 and p21 (in addition to their ability to inhibit cdk2 and bind E2F) is that both proteins have amino-terminal and carboxy-terminal cyclin-binding motifs. Although the exact molecular details behind cyclin-binding and kinase inhibition is not yet known for either protein, it is interesting to speculate that they use identical mechanisms to inhibit cdk2 and cell growth.

In the future, experiments designed to examine p130/cyclin/cdk2 and p107/cyclin/cdk2 complexes in normal and cancerous mammary cell lines may eventually help to elucidate whether a kinase titration model for cell cycle progression is indeed correct. For example, we have demonstrated (section 1 above) that p21 appears to limit cdk activity in the cell, and S phase entry could be provoked in part when an excess of cyclin A/cdk2 is reached relative to this CKI. Our experiments with p107 and p130 clearly indicate that we must expand the list of cellular CKIs to include these proteins. As discussed in section 1C above, we are continuing to ask to what extent the overall cyclin-dependent kinases activity contributes to cell cycle status in normal and transformed cells.

We have recently begun to examine whether the p21-E2F, p107/p130-E2F, and p107/p130-CDK pathways are intact in mammary carcinoma cells as well. For this purpose, we have begun largescale growth of several cell lines, including those listed in previous sections.

7. CONCLUSIONS

We have, for the first time, begun to shed light on the molecular architecture and activity of endogenous p21 complexes. Previous work suggested that p21 could participate in complexes that contained an active kinase (Zhang et al., 1994; Harper et al., 1995). However, many of these prior studies were performed with a single polyclonal anti-p21 antibody. Moreover, no attempt had been made to compare the specific activity of kinase complexes containing p21 with those lacking this CKI; only absolute levels of kinase activity were measured. We chose to develop and systematically characterize a panel of monoclonal antibodies against p21. We believe that these MAbs will be invaluable in our continued efforts to study the configuration and biochemical activities of p21 complexes in normal and transformed cells.

To this end, we initiated a thorough biochemical characterization of p21-containing complexes in normal breast and breast carcinoma cell lines. We have found that cyclin A/cdk2 associated with p21 in vivo is largely inactive, challenging previous findings (Zhang et al., 1994; Harper et al., 1995). It is important to point out several differences between our study and others. First, we have not studied complexes assembled in vitro or by over-expression through transient transfection. Such experiments could be misleading if complexes with incorrect stoichiometries are assembled. Instead, we have focused exclusively on cellular complexes. In addition, we have compared the specific activity of similar amounts of cyclin A/cdk2 in a p21-bound and un-bound state using immunoblotting and histone H1 kinase assays. Lastly, we have not measured the kinase activity associated with cyclin D/cdk4/p21 or cyclin E/cdk2/p21 because of the low abundance of kinase activity associated with these cyclins in the cell lines we have studied (not shown). Active native complexes containing cyclin D and p21 have been noted (LaBaer et al., 1997).

Generation of monoclonal antibodies with unique properties has also proven extremely useful for studying the changes in the composition of endogenous p21 complexes during traversal of G1 phase. For example, antibody CP55 appears to recognize "free" p21, that is, p21 that migrates close to the expected size of 21 kDa on a sizing column. We found that such an antibody detects abundant amounts of "free" p21 early in G1, but this species disappears as cells progress toward S phase. We propose that excessive p21 may limit kinase activity early in G1, but as the amount of cyclin A/cdk2 exceeds that of p21, S phase entry could be triggered. We are currently attempting to test this idea using additional normal and breast cancer cell lines.

We have also demonstrated that in addition to the p21/p27/p57 and p15/INK4 families of proteins, two members of the pRB protein family can function as physiological CKIs. Because p107 and p130 inhibit a subset of the kinases inhibited by p21, it is possible that p21 nullizygous mice may not develop tumors due to redundancy of function. This hypothesis will likely be tested by other groups by producing a mouse nullizygous for different combinations of p107/p130 and p21. Finally, since both p21 and p107/p130 target identical E2F family members, p21 and p107/p130 growth restraining pathways may be interrelated in two way: they may both participate in growth suppression by inhibition of kinase activity and activity of the E2F S-phase-promoting transcription factor.

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9. APPENDIX (see attached pages)

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Activity and Nature of $p21^{WAF1}$ Complexes During the Cell Cycle

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Classification: Biological Sciences (Biochemistry)

Abbreviations: Cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; PCNA, proliferating cell nuclear antigen

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Abstract

Elevated levels of the $p21^{WAF1}$ (p21) cyclin-dependent kinase inhibitor (CKI) induces growth arrest. We have characterized a panel of monoclonal antibodies against human p21 in an effort to understand the dynamic regulatory interactions between this and other cellular proteins during the cell cycle. The use of these reagents has allowed us to address several important, yet unresolved, issues concerning the biological activity of p21, including the potential kinase activity of complexes that associate with this CKI. We have found that the kinase activity of cyclin A/Cdk2 associated with p21 is significantly lower than that of cyclin A/Cdk2 free of p21, suggesting that p21 abolishes its activity in vivo, and the use of multiple antibodies has enabled us to begin the study of the molecular architecture of p21 complexes in vivo. In addition, we found that human fibroblasts released from a quiescent state display abundant amounts of p21 devoid of associated proteins ("free" p21), the levels of which decrease as cells approach S phase. Cyclin A levels increase as the amount of monomeric p21 decreases, resulting in an excess of cyclin A/Cdk2 complexes that are not bound to, or inactivated by, p21. Our data strengthen the notion that the G1-to-S phase transition in human fibroblasts occurs when the concentration of cyclin A/Cdk2 surpasses that of p21.

Introduction

Mammalian cell cycle progression is governed by an interplay between positive and negative regulatory factors. Cyclin-dependent kinases (Cdks), composed of regulatory cyclin and catalytic Cdk subunits, are activated in a periodic manner to promote cell cycle transitions, while another family of proteins, the cyclin-dependent kinase inhibitors (CKIs), acts in a negative fashion by extinguishing the activity of Cdks (reviewed in 1). Biochemical studies have shown that one CKI, p21 (also known as WAF1/Cip1/Sdi1/Cap20, henceforth referred to as p21), binds tightly to the G1 and S phase kinases, cyclin E/Cdk2, cyclin D/Cdk4, and cyclin A/Cdk2 and effectively inhibits their activity, while p21 is a relatively poor inhibitor of the G2/M phase kinase, cyclin B/Cdc2 (2-4). Additional studies have indicated the presence of PCNA, cyclin, and Cdk binding domains in p21 (5-8), and crystallographic studies suggest a mechanism for kinase (and growth) inhibition by the related CKI, $p27^{Kip1}$ (9). Inhibition of DNA replication through interactions with PCNA is thought to constitute a second mechanism by which p21 can act as a growth suppressor (ref. 1 and references therein). One unexpected result of in vitro studies, however, was the finding that sub-stoichiometric levels of p21 did not abolish kinase activity, but rather facilitated assembly of cyclins with their Cdk partner, whereas higher amounts of p21 were inhibitory (4, 10). Moreover, kinase activity associated with endogenous p21 has also been detected (4, 10, 11).

Nevertheless, a rigorous comparison between the specific activity of endogenous kinase complexes associated with, and devoid of, p21 has been lacking. In an effort to understand how p21 regulates the activity of associated kinases during cell cycle progression, we have begun to characterize a panel of monoclonal antibodies raised against this protein. These antibodies recognize distinct p21-associated complexes through a diverse array of epitopes

on the p21 protein and reveal structural details of such complexes in a more physiological setting than previous analyses. Using these reagents, we have also studied the composition and kinase activity of p21 complexes. These experiments suggest that p21 modulates kinase activity in vivo, and the abundance of this CKI may contribute to the timing of the G1/S transition.

Materials and Methods

Antibodies and epitope mapping. Antibodies were produced against recombinant human p21 as described previously (12). The epitope recognized by each was mapped using a synthetic peptide array (Pepspots; Jerini BioTools, GMBH, Germany) containing 77 peptides spanning the entire sequence of human p21. All peptides were 13-mers that differed sequentially by two carboxy-terminal amino acids and were covalently immobilized at their carboxy-terminus to a nitrocellulose membrane as separate spots. Mapping was carried out according to the manufacturer's instructions. Briefly, the antibody to be mapped was first incubated with the peptide array for 3-5 hours at 4°C. After extensive washes, the bound antibody was transferred to a second piece of nitrocellulose and processed using standard blotting techniques.

Other antibodies used in this study included anti-cyclin A (BF683), anti-cyclin B (GNS1), anti-cyclin E (HE12), and polyclonal anti-cyclin D1, kindly provided by S. Shiff, E. Harlow, and L. Zukerberg. Additional antibodies against cyclin A (H432), Cdk2 (M2), Cdk4 (C-22), E2F-4, p21 (C-19G), Cdc2 (17), and PCNA (PC10) were purchased from Santa Cruz Biotechnology, Inc.

Immunoprecipitations, kinase assays, and western blotting. Whole cell extracts were prepared using ELB buffer containing 50 mM HEPES, pH 7, 250 mM NaCl, 5 mM EDTA, 0.1 % NP 40, 10 % glycerol, 0.5 mM DTT, 0.5 mM AEBSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 10 mM NaF, and 50 mM β-glycerophosphate. Cell extracts (300 μg of total protein) were pre-cleared with protein A sepharose and normal rabbit serum and immunoprecipitated by incubation with antibody-conjugated beads. After washes with ELB and kinase buffer, the beads were halved and subjected to western blot analysis and kinase assays. For immunoprecipitation of ^{35}S-labeled proteins, WI38 cells were

incubated for four hours in ^{35}S-methionine, lysed in ELB, and pre-cleared prior to incubation with specific antibody.

Western blots were analyzed by NIH Image version 1.61. Kinase assays were performed as described previously (14), and the results were quantitated using a PhosphorImager. We calculated the specific activity of Cdk2 complexes immunoprecipitated by anti-cyclinA and anti-p21 (CP2) as follows: Specific activity of cyclin A/Cdk2 = histone H1 kinase activity (PhosphorImager units)/Cdk2 protein (arbitrary densitometry units). Recombinant human p21 was produced in bacteria and purified as described previously (15).

Denaturing versus native immunoprecipitation. To unfold and dissociate all protein complexes, 300 μg of WI38 whole cell extracts were first treated with 8 M urea in ELB buffer for 30 min at room temperature. Samples were then diluted 20-fold with buffer containing indicated antibody-bead conjugates and rocked for 1 h at 4°C. Control samples either contained a final concentration of 0.4 M urea (to match conditions of re-folded samples) or were untreated but normalized for protein concentration. Bound proteins were separated on a 12% SDS-PAGE gel and analyzed by western blotting using anti-p21 antibody (CP36).

Cell lines and cell cycle synchronization. WI38 human fibroblasts and the SV40-transformed derivative WI38-VA13 were obtained from ATCC. WI38 cells at a passage number of 15-17 grown to about 70% confluence in DMEM with 10% fetal bovine serum were serum-starved by incubation in medium containing low serum (0.2%) for three days. Under these conditions, greater than 85% of the cells were arrested at G0/G1 as determined by FACS analysis (FACScan Analyzer, Becton Dickinson) after propidium iodide staining.

Size exclusion chromatography. A 10 x300 mm Sephacryl S-300 (Pharmacia Biotech) column was used to analyze the size of p21-containing protein complexes. The column was equilibrated in, and developed with, 50 mM HEPES, pH 7.4, containing 250 mM NaCl, 1 mM DTT, and 1 mM PMSF at 4°C. The flow rate was 0.5 ml/min, and 0.7 ml fractions were collected. The column was calibrated with the following proteins: cytochrome c (12.4 kDa); carbonic anhydrase (29 kDa); bovine serum albumin (67 kDa); lactate dehydrogenase (140 kDa); catalase (232 kDa); apoferritin (443 kDa); and thyroglobulin (669 kDa).

Results and Discussion

Characterization of anti-p21 antibodies. A panel of monoclonal antibodies raised against native, recombinant human p21 purified from bacteria has been described (12). We mapped precisely the epitopes recognized by each antibody using a peptide array as described in the Materials and Methods section (Fig. 1A). Each of these antibodies are specific for p21, although one (CP36) cross-reacts weakly with recombinant p27 and p57 on western blots and immunoprecipitates these recombinant proteins with very low efficiency (12).

Initially, we immunoprecipitated whole cell extracts derived from asynchronous W138 human fibroblasts with anti-p21 antibodies and detected p21-associated proteins by western blotting (Fig. 1B). For the purposes of comparison, we also included in our studies an antibody purchased commercially (C19) that recognizes the overlapping PCNA-binding/carboxy-terminal cyclin-binding domains of p21 (residues 146-164). We demonstrated that several antibodies (CP2, 59, 68) co-precipitated p21 with the full complement of known cell cycle regulatory proteins, including cyclin A, cyclin B, cyclin D, cyclin E, Cdk2, Cdk4, Cdc2, and PCNA (16). As expected from previous studies, very low levels of cyclin B/Cdc2 associated with p21 in vivo, perhaps as a consequence of the relatively low affinity between this kinase and p21 (2, 4, 17).

Interestingly, several antibodies recognize epitopes that overlap with important functional domains in p21, and they were able to immunoprecipitate subsets of the above complexes (12). For example, CP36 recognizes an epitope in p21 that precisely corresponds to an amino-terminal cyclin-binding domain found in the p21 family of proteins and in the retinoblastoma protein (pRB) family members, p107 and p130 (8, 15, 18). This cyclin-binding domain, referred to variously as the LFG, RXL, or Cy motif (henceforth, Cy domain) is present near the amino- and carboxy-termini of p21. CP36

immunoprecipitates contained cyclin A and Cdk2 but none of the other cyclins, Cdks, or PCNA (Fig. 1B). Significantly, CP36 immunoprecipitates much lower amounts of p21 and Cdk2 than CP2, 59, or 68, although it can retrieve similar amounts of cyclin A, suggesting that the antibody strictly recognizes cyclin A/Cdk2/p21 complexes. Notably, no PCNA is co-precipitated with this complex, suggesting the existence of cyclin A/cdk2/p21 complexes lacking PCNA. We have also been able to distinguish these complexes lacking PCNA from others containing cyclin D/Cdk4, p21, and PCNA by ion exchange chromatography (data not shown). These studies, together with a previous one (8), suggest that cyclins D and E associate with p21 in vivo principally through the amino-terminal cyclin-binding domain recognized by CP36, while cyclin A is also able to associate with this inhibitor through the carboxy-terminal cyclin-binding domain or indirectly via a Cdk2 bridge, which can independently associate with p21 (5-8). Since the carboxy-terminal cyclin-binding (Cy) domain overlaps with the PCNA-binding domain in p21, these results raise the possibility that cyclin A can successfully compete with PCNA for binding to this site in vivo.

Another antibody, CP55, recognizes an epitope in p21 (residues 69-78) corresponding to a region in $p27^{KIP1}$ known to form a 3_{10} helix that is buried deeply in the catalytic cleft of Cdk2 in the cyclin A/Cdk2/p27 ternary complex, the structure of which was solved crystallographically (9). Intriguingly, immunoprecipitates of CP55 were devoid of all other cyclin and Cdk proteins as detected by western blotting (Fig. 1B). Occasionally, small amounts of PCNA and Cdk4 were co-precipitated with p21, but in no cases were any cyclins detected. These and other experiments (described below) suggest that CP55 is able to recognize a form of p21 we have termed "free" p21.

To visualize the purity of complexes that co-purify with p21, we immunoprecipitated p21 from exponentially growing WI38 cells that were metabolically labeled with ^{35}S-methionine. In these experiments, immunoprecipitates were highly enriched for the same p21-associated proteins revealed by western blotting (Fig. 1C). Few contaminating proteins were detected in most immunoprecipitates, although certain ones contained unidentified proteins that await further characterization. Importantly, these experiments confirmed the specificity of our anti-p21 antibodies for this CKI family member, since we did not observe the co-precipitation of either p27 or p57 from WI38 cell lysates (data not shown). The purity of these complexes and the absence of the two p21-related proteins was an important consideration for subsequent functional studies concerning the kinase activity of p21 complexes (see below), and they suggest the usefulness of these antibodies in purifying distinct complexes to homogeneity for future activity studies.

The inability of CP36 and CP55 to recognize a subset of p21-associated proteins was not due to reduced affinities between these antibodies and p21 because each of the five monoclonal antibodies immunoprecipitated equal amounts of purified, recombinant p21 produced in bacteria (Fig. 1B). Furthermore, when we examined the ability of each of our antibodies to recognize native versus denatured p21-containing complexes, we noticed distinct differences between the two groups of antibodies (Fig. 1D). Whereas CP2, CP59, and CP68 recognized equivalent amounts of p21 before and after denaturation of cell lysates with 8 M urea, both CP36 and CP55 immunoprecipitated significantly more p21 after denaturation and renaturation in the presence of antibody (compare lanes "N" and "R").

Taken together, these data suggest that regions recognized by CP36 (Cy motif) and CP55 (residues that potentially form a Cdk inhibitory helix) are not exposed in vivo. These

experiments for the first time begin to elucidate the molecular architecture of p21 complexes in vivo and are in agreement with previous in vitro and structural studies.

Cyclin/Cdk complexes associated with p21 are inactive. One important unresolved issue regarding p21 concerns the apparent ability of this cyclin-dependent kinase inhibitor to associate with active cyclin/Cdk complexes in vitro and in vivo (4, 10, 11). Counter-balancing these studies are others suggesting that depletion of p21-containing complexes does not decrease the overall histone H1 kinase activity associated with cyclin A (17). It has been postulated that the presence or absence of p21-associated kinase activity depends upon the stoichiometry of p21 molecules bound to the kinase: low levels of p21 actually stimulate assembly of associated kinases, whereas incorporation of a second molecule inhibits activity (4, 10, 11).

In an effort to resolve these contradictory observations, we have measured the histone H1 kinase activity associated with p21 in vivo using our panel of antibodies. We reasoned that this approach had the following advantages over previous analyses. First, we employed at least six different monoclonal antibodies recognizing epitopes spanning the p21 protein. This reduces the likelihood that kinase activity was altered by antibody binding to the complex. Second, the use of antibodies that immunoprecipitate all known associated cyclin/Cdk complexes as well as subsets of complexes (for example, CP36), suggests that we can study both the total Cdk2 kinase activity of all associated complexes as well as that of the cyclin A/Cdk2 complex in isolation. Third, the use of high-affinity antibodies results in immunoprecipitates that have been greatly enriched for p21-containing complexes (Fig. 1C) and are thus less likely to contain contaminating kinase activities. Finally, a meaningful estimate of p21-associated kinase activity requires not only a measurement of absolute kinase activity but also the specific activity of cyclin A/Cdk2 complexes in a free state and when bound to p21.

Figure 2 shows that anti-p21 immunoprecipitates displayed very low levels of histone H1 kinase activity detectable only upon prolonged autoradiographic exposure. CP36 immune complexes had the highest amounts of associated kinase activity (12), but even in this case, the specific activity of the ternary complex (Materials and Methods) was significantly reduced relative to cyclin A/Cdk2 that was immunoprecipitated directly from extracts or from p21-depleted extracts (lanes 13-14; for quantitation, see Fig. 3F). Moreover, cyclin A immunoprecipitates had similar amounts of histone H1 kinase activity whether or not p21 had been previously depleted, in agreement with a recent report (17). Similar results were obtained when extracts were immunoprecipitated with anti-Cdk2 antibodies or an unrelated anti-cyclin A monoclonal antibody, rendering unlikely the possibility that anti-cyclin A antibodies artificially caused the activation of kinase complexes (data not shown). We have also observed similar differences in the specific activities of binary and ternary complexes in the human breast cancer cell line MCF-7 (data not shown).

We conclude that in an asynchronous population of human fibroblasts, cyclin A/Cdk2 complexes associated with p21 are inactive. Here,we have for the first time compared the specific activities (in contrast to absolute kinase activity alone) of native cyclin A/Cdk2 complexes by immunoprecipitation with multiple antibodies. Although these experiments do not allow us to discriminate between complexes that may contain one or more molecules of p21--the latter being the proposed inhibitory form of p21 (10)-- they nevertheless indicate the complete loss of activity of Cdks upon association with p21 in vivo. Recent experiments with p21 -/- mouse embryo fibroblasts, in which Cdk2 (but not Cdk4) activity is enhanced relative to the wild-type control, support such an in vivo inhibitory activity for p21 (19).

Dynamics of p21 interactions with cyclin/Cdk2 during the cell cycle. In light of the above findings with asynchronous cells suggesting the near-complete inhibition of Cdk2 associated with p21, we considered the possibility that p21 complexes with cyclin-dependent kinases might display activity at specific stages of the cell cycle and that a survey of an asynchronous population would preclude a measurement of such activity. To this end, WI38 cells were made quiescent by serum starvation and re-stimulated to enter the cell cycle by addition of 10% serum. Serum deprivation resulted in the arrest of a majority of cells (greater than 85%) as determined by FACS analysis (Fig. 3A). The cells re-entered the cell cycle upon serum addition, entering S phase between 16 and 20 hours after re-stimulation. The largest fraction of cells were in S phase 24 hours after re-stimulation. Extracts of samples collected at each time point during serum deprivation and re-stimulation were subjected to direct western blotting to determine the total amounts of cyclin A, Cdk2, and p21 present during cell cycle re-entry (Fig. 3C). As expected from previous studies (20-22), the abundance of p21 protein does not fluctuate dramatically during the time course, although p21 levels did increase by approximately two-fold upon serum stimulation (compare 0 and 4 h time points). In contrast, cyclin A levels rose sharply as cells entered S phase, peaking between 20-24 hours post-stimulation (data not shown).

To estimate the level of cyclin A/Cdk2 kinase activity associated with p21 as cells exited G0 and progressed through G1 and S phases, we performed the sequential immunoprecipitations outlined in Fig. 3B. Western blotting was performed at each stage to estimate the levels of each protein of interest. Since the ultimate goal of these experiments was to determine the kinase activity of cyclin A/Cdk2/p21 complexes and cyclin A/Cdk2 that was completely devoid of p21, we initially performed immunoprecipitations on extracts from each time point with CP55 to inspect "free" p21

levels and to remove this factor from further activity considerations. As expected from our initial studies, CP55 immunoprecipitated p21 to the exclusion of both cyclin A and Cdk2 (Fig. 3D). Remarkably, we observed a vast excess of "free" p21 in quiescent and early G1 phase cells. Between 8 and 12 hours post-stimulation, however, "free" p21 levels dropped precipitously, although the total amount of p21 during this time remained largely unchanged (compare 4-12 hour time points in panels C and D). Furthermore, this cell cycle-dependent immunoprecipitation of p21 by CP55 indicates that this antibody is not simply disrupting p21 complexes and thereby artificially creating "free" p21.

After depletion of "free" p21 from extracts at each time point with CP55, we subsequently immunoprecipitated the same extracts with CP2, which recognizes all known p21-associated cyclin/Cdk complexes (see Fig. 1). Although robust amounts of p21 and Cdk2 were precipitated throughout the time course, cyclin A was not detected until approximately 12 hours after serum addition, as expected from western blots of total cell lysate (Fig. 3E). However, association between this newly synthesized cyclin A/cdk2 and p21 resulted in negligible levels of histone H1 kinase activity, again suggesting that Cdk2 complexes are inhibited by association with p21 in vivo, irrespective of cell cycle position.

Successive rounds of immunoprecipitation with CP2 led to depletion of p21 from extracts, as determined by western blotting (Fig. 2 and Fig. 3F). Next, the use of these depleted extracts allowed us to estimate the abundance and kinase activity of cyclin A/Cdk2 complexes lacking p21 as cells traversed the G1 phase. Immunoprecipitation of such extracts with anti-cyclin A antibodies revealed the presence of cyclin A/Cdk2 16 and 20 hours after serum stimulation, and as expected, this kinase was not associated with p21 (Fig. 3F). Thus, most, but not all, of the cyclin A/Cdk2 is associated with p21 in these cells. When we compared the specific kinase activity of residual cyclin A/Cdk2 not associated with p21 (Fig. 3F, 16 and 20 h time points) with that of the cyclin A/Cdk2/p21

ternary complex (Fig. 3E, 16 and 20 h time points), we noticed a striking difference. Although there was considerably less cyclin A and Cdk2 in binary complexes as compared to the amount present in the ternary complex obtained by immunoprecipitation with CP2, the amount of kinase activity in the former complex was much greater. Indeed, when we calculated the specific Cdk2 activity of both complexes by performing PhosphorImager analysis and densitometric scanning of corresponding autoradiograms and western blots (see Materials and Methods), we found that the activity of the cyclin A/Cdk2 complex was 166-fold and 158-fold higher than that of the ternary complex at 16 and 20 hours post-stimulation, respectively.

We were concerned about the possibility that the above findings may have resulted from artificially elevated levels of p21 induced by serum stimulation of quiescent cells (20, 21, 23). To alleviate these concerns, we induced quiescence in WI38 cells by a second method, namely, contact inhibition. This treatment resulted in growth arrest of a substantial fraction of cells (greater than 80%), and high levels of "free" p21 similar to those described above were observed exclusively at early time points after release from contact inhibition (data not shown). This suggests that the existence of "free" p21 may be an intrinsic feature of early G1 cells, although confirmation will require the testing of additional cell lines using similar and unrelated synchronization techniques.

From these experiments, we can draw several conclusions. First, although total p21 levels do not change dramatically during the cell cycle, the amount of "free" p21 (i.e., p21 that is not associated with cyclin/Cdk complexes) is high in early G1 but drops sharply as cells progress toward S phase. From these data, we surmise that excess p21 ensures that cyclin/Cdk complexes are inactive in early G1. As cells progress through G1, cyclin A levels rise, and the levels of cyclin A/Cdk2 surpass those of p21, providing the impetus for the onset of S phase. Thus, our data support a threshold hypothesis for p21, which had

been proposed based on in vitro experiments, in which the ratio of Cdk to p21 determines enzymatic activity (4). However, in contrast with previous findings, we do not detect significant histone H1 kinase activity associated with p21 during cell cycle progression from G0 to S phase, when cyclin A/Cdk2 and cyclin A/Cdk2/p21 complexes are compared.

Estimating the size of "free" p21. The preceding experiments indicated that one antibody, CP55, was uniquely able to recognize p21 to the exclusion of all other cell cycle regulators. To rule out the possibility that CP55 immunoprecipitated a complex of other proteins not detected in our western blotting analysis, we estimated the size of p21 complexes in early G1 (4 h after serum restimulation) and S phases (24 h after serum stimulation) by subjecting extracts of WI38 cells to S-300 gel filtration chromatography (Fig. 4). Extracts from early G1 cells contained both "free" p21, which migrated at a position (between the 12 kDa and 29 kDa markers) predicted for a monomer, and larger complexes (between 67 kDa and 443 kDa markers) that contained p21 in association with cyclin D/Cdk4, cyclin E/Cdk2, and PCNA. Of these two pools, CP55 was solely capable of immunoprecipitating the low molecular fraction between 12 and 29 kDa in size. The p21 fraction immunoprecipitated by CP55 co-migrates exactly with recombinant p21 purified from bacteria (data not shown), suggesting that a form of p21 free of interacting proteins was recognized by this antibody. In striking contrast, p21 is largely confined to high molecular weight complexes in extracts from S phase cells.

We tested whether mixing extracts from cells stimulated for 4h would inhibit cyclin A/Cdk2 kinase activity found in 24 h cell extracts, and this was indeed the case (data not shown), suggesting that this CKI was at least partly responsible for the inhibition of kinase activity in early G1 cells.

In summary, we have described a set of antibodies raised against p21 that have allowed us to document the low specific kinase activity associated with this CKI in vivo. These findings contrast with previous ones, some of which relied on reconstitution of ternary complexes with recombinant proteins, to show that kinase complexes with p21 retained activity (4, 10). Our experiments differ in several important ways. First, we have used multiple monoclonal antibodies to study endogenous p21-associated histone H1 kinase activity, whereas previous studies relied on polyclonal sera. Second, we have examined the specific activity of p21-associated kinase complexes, rather than absolute kinase activity, and compared it with cyclin A/Cdk2 complexes lacking p21. It is important to point out that we have not addressed the specific kinase activity of p21 complexes with cyclin D and cyclin E. Such experiments may be informative in light of recent data with a p27-inducible cell line in which it was shown that Cdk2, but not Cdk4, was inhibited by elevated p27 (24). Nevertheless, our novel findings regarding "free" p21 in early G1 phase lend physiological support to a threshold hypothesis for cell cycle regulation by the p21 protein.

Figure Legends

Fig. 1.

Characterization of anti-p21 antibodies. (A) Schematic representation of functional domains of p21 and the epitopes recognized by anti-p21 antibodies. Epitopes were mapped using a peptide array as described in Materials and Methods. On the basis of this analysis, we mapped the antibody recognition to the following epitopes: residues 5-13 (CP2), residues 19-25 (CP36), residues 35-43 (CP50), residues 69-78 (CP55), residues 96-104 (CP59), and residues 95-102 (CP68). One antibody, CP50, failed to immunoprecipitate endogenous p21 and was not characterized further. (B) 300 μg of asynchronous WI38 cell extracts were immunoprecipitated with different anti-p21 antibodies as indicated. The first lane ("IN") represents 10% of input protein loaded directly. The last lane shows a control using anti-flu hemagglutinin (HA) monoclonal antibody, 12CA5. For comparison, recombinant p21 (rp21; 3 ng) from bacteria was immunoprecipitated. (C) Immunoprecipitations of extracts of WI38 cells metabolically labeled with ^{35}S-methionine using the indicated antibodies were resolved by SDS-PAGE and visualized by fluorography. The identity of associated proteins was assigned by parallel immunoprecipitations with antibodies against each of the indicated proteins. Arrowheads and asterisks indicate non-specifically precipitated (found in anti-HA immunoprecipitates) and unidentified proteins, respectively. (D) The epitopes recognized by CP36 and CP55 are buried in cellular p21-containing complexes. 300 μg of asynchronous WI38 cell whole extracts were treated without urea (native or N), or with 0.4 M (N') or 8 M (refolding or R) urea as described in Materials and Methods. Samples were then diluted 20-fold with buffer (N and R) or 0.4 M urea (N') and immunoprecipitated and western blotted. HA is a control using antibody 12CA5.

Fig. 2.

The p21/cyclin A/Cdk2 ternary complex is significantly less active than the cyclin A/Cdk2 complex in vivo. Whole cell extracts from exponentially growing WI38 cells were split into two equivalent samples. One half was left untreated while the other was treated with three rounds of CP2-conjugated beads to deplete p21-containing complexes. Immunoprecipitations were performed on both depleted (+) and non-depleted (-) samples using different antibodies as indicated. The immunoprecipitated samples were split again and tested by western blotting using anti-p21, anti-cyclin A, and anti-Cdk2 antibodies and in histone H1 kinase assays.

Fig. 3

Excess "free" p21 exists exclusively during early G1 phase. (A) WI38 cells were synchronized by serum deprivation, and the cell cycle profile determined by FACS analysis is shown. (B) Whole cell extracts derived from WI38 cells were analyzed directly or by sequential immunoprecipitation according to the scheme shown. (C) 20 μg of extract from each stage were loaded directly and detected by western blotting to reveal the total amounts of cyclin A, Cdk2, and p21. (D) 300 μg of protein were immunoprecipitated using CP55 to detect free p21 at each cell cycle time point (the first round of three consecutive depletions is shown). (E) Supernatants after three rounds of depletion of free p21 by CP55 were subjected to CP2 immunoprecipitation to detect CyclinA/Cdk2/p21 ternary complexes during the cell cycle. Histone H1 kinase activity was measured in parallel. (F) Supernatants after the immunoprecipitation in panel E were subjected to immunoprecipitation using anti-cyclin A antibody-conjugated beads to detect the remaining cyclinA/Cdk2 complex. Relative western blot exposure times are the same for each part of the figure. Histone H1 kinase activity was measured in parallel.

Fig. 4

Free p21 in WI38 cells 4 hours after serum re-stimulation revealed by sizing column chromatography. An S-300 column was used to separate p21 and its complexes as described in Materials and Methods. The elution of calibration standards is indicated at the top. 500 μg of protein derived from extracts of cells 4 h or 24 h after serum stimulation were loaded on the column. Fractions were collected, halved, and immunoprecipitated with either CP2 or CP55 as labeled. The samples were analyzed by western blotting to reveal p21 and its complexes.

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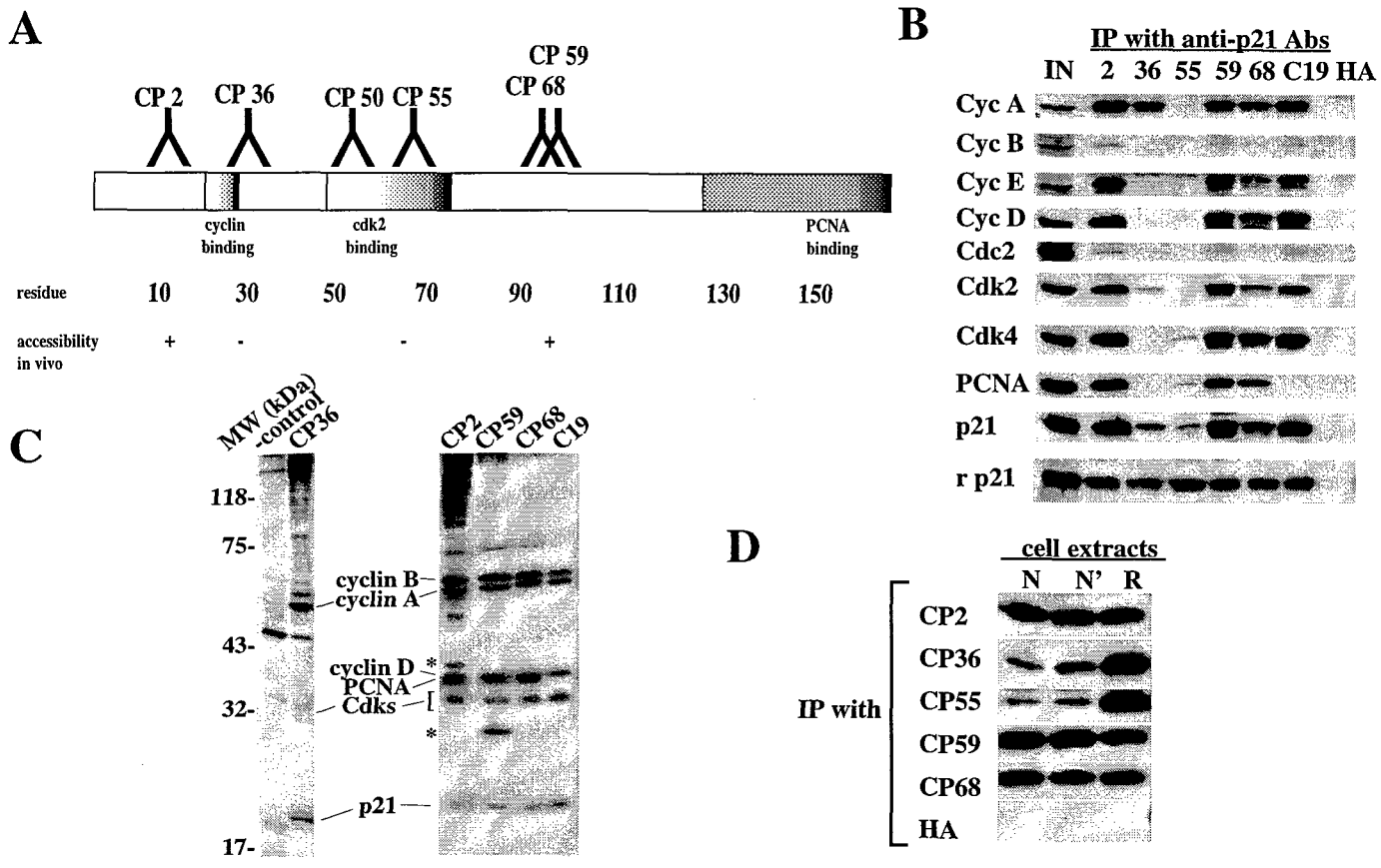
Figure 1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PCNA binding
residue
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110
130
150
accessibility in vivo
+
-
-
+
B
IP with anti-p21 Abs
IN
2
36
55
59
68
C19
HA
Cyc A
Cyc B
Cyc E
Cyc D
Cdc2
Cdk2
Cdk4
PCNA
p21
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C
MW (kDa)
-control
CP36
CP2
CP59
CP68
C19
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17-
cyclin B
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cyclin D
PCNA
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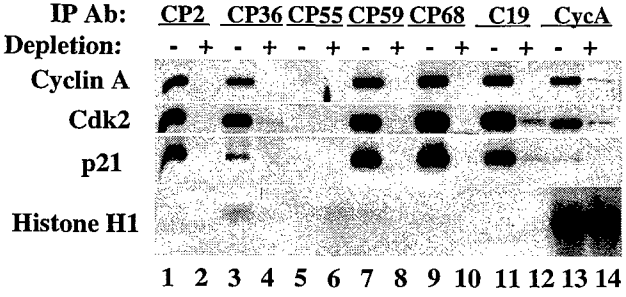
Figure 2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CP36
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CP59
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C19
CycA
Depletion:
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Cdk2
p21
Histone H1
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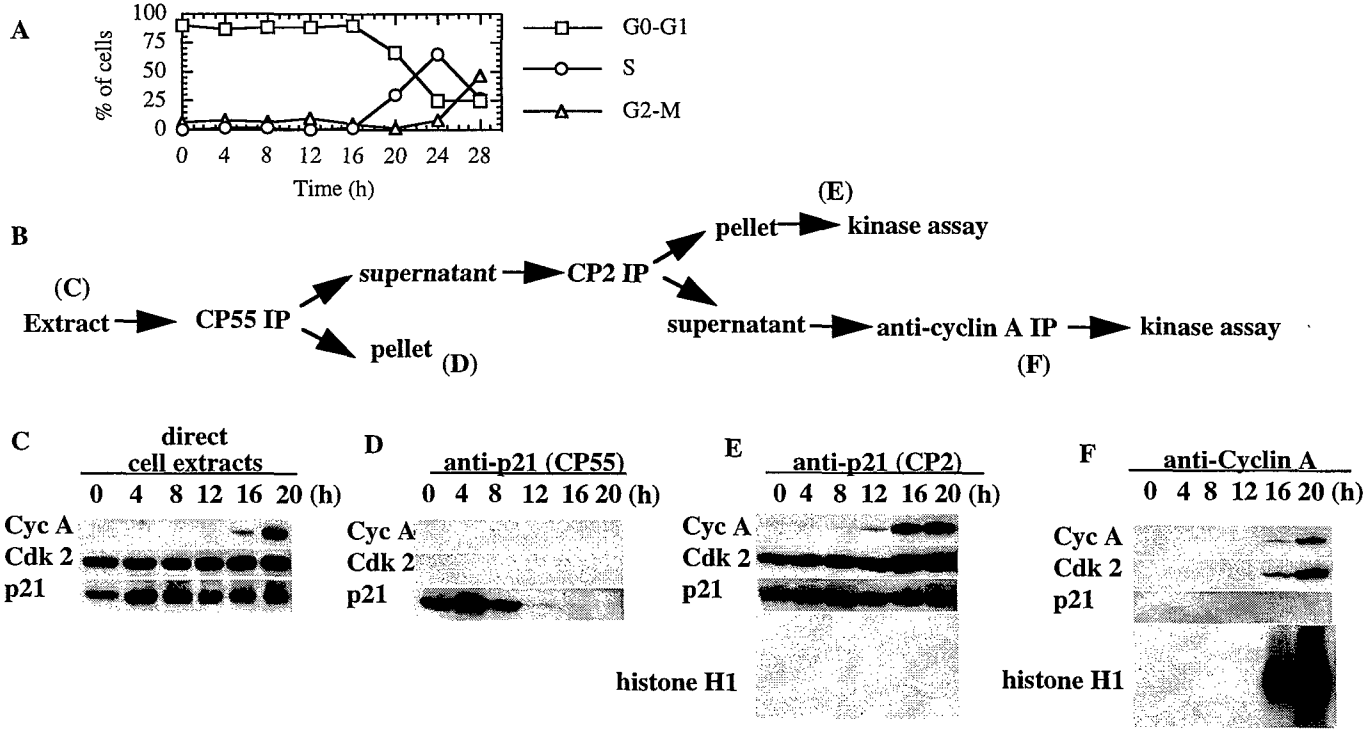
Figure 3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100
75
50
25
0
% of cells
0 4 8 12 16 20 24 28
Time (h)
G0-G1
S
G2-M
B
(C)
Extract
CP55 IP
supernatant
pellet
(D)
CP2 IP
(E)
pellet
kinase assay
supernatant
anti-cyclin A IP
(F)
kinase assay
C
direct
cell extracts
0 4 8 12 16 20 (h)
Cyc A
Cdk 2
p21
D
anti-p21 (CP55)
0 4 8 12 16 20 (h)
Cyc A
Cdk 2
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Cyc A
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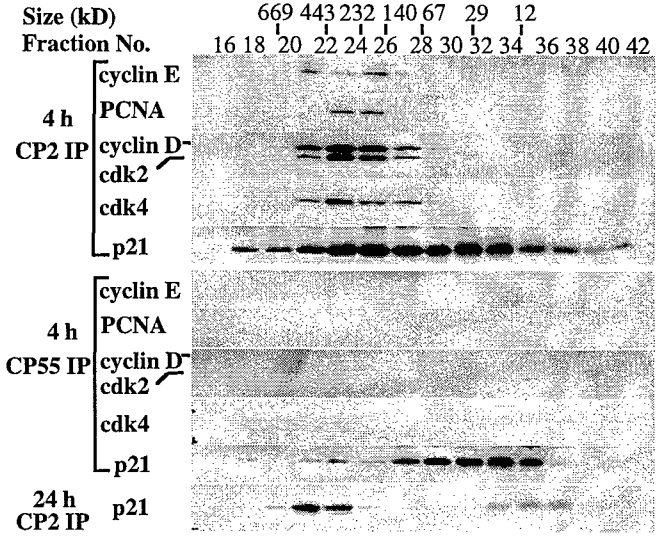
histone H1

Figure 4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Dual Cyclin-Binding Domains Are Required for p107 To Function as a Kinase Inhibitor

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The retinoblastoma (pRB) family of proteins includes three proteins known to suppress growth of mammalian cells. Previously we had found that growth suppression by two of these proteins, p107 and p130, could result from the inhibition of associated cyclin-dependent kinases (cdks). One important unresolved issue, however, is the mechanism through which inhibition occurs. Here we present in vivo and in vitro evidence to suggest that p107 is a bona fide inhibitor of both cyclin A-cdk2 and cyclin E-cdk2 that exhibits an inhibitory constant (K_i) comparable to that of the cdk inhibitor p21/WAF1. In contrast, pRB is unable to inhibit cdks. Further reminiscent of p21, a second cyclin-binding site was mapped to the amino-terminal portions of p107 and p130. This amino-terminal domain is capable of inhibiting cyclin-cdk2 complexes, although it is not a potent substrate for these kinases. In contrast, a carboxy-terminal fragment of p107 that contains the previously identified cyclin-binding domain serves as an excellent kinase substrate although it is unable to inhibit either kinase. Clustered point mutations suggest that the amino-terminal domain is functionally important for cyclin binding and growth suppression. Moreover, peptides spanning the cyclin-binding region are capable of interfering with p107 binding to cyclin-cdk2 complexes and kinase inhibition. Our ability to distinguish between p107 and p130 as inhibitors rather than simple substrates suggests that these proteins may represent true inhibitors of cdks.

Orderly progression through the cell cycle requires the orchestration of growth-promoting and -restraining signals. The cyclin-dependent kinases (cdks) are believed to constitute some of the most important factors driving proliferation of eukaryotic cells (40). These proteins function by phosphorylating substrates required to affect each transition through the cycle. The growth-promoting influences of the cdks are counterbalanced by two groups of cdk inhibitors (CKIs), which include at least seven members. One group includes p21 (also known as WAF1/Cip1), p27 (Kip1), and p57 (Kip2), while the second group is comprised of p15, p16, p18, and p19 (also known as INK4 proteins; reviewed in reference 36). These low-molecular-weight inhibitors were classified based on sequence homology and the kinases inhibited by each. The first group is thought to bind primarily to those kinases involved in G_1 and S phase progression (i.e., kinases associated with cyclins A, D, and E), while the second group exclusively inhibits kinases associated with cyclin D.

The retinoblastoma tumor suppressor (pRB) and the related proteins p107 and p130 comprise another class of proteins involved in limiting cell cycle progression. pRB is thought to control entry into S phase in part by repressing the activity of E2F, a transcription factor known to promote proliferation (40). Another member of the pRB family, p107, regulates cell cycle progression by at least two distinct mechanisms (38, 42). p107 can also inhibit the activity of the E2F transcription factor (34, 45). In addition, p107 can interact with the cdks cyclin A-cdk2 and cyclin E-cdk2 through a second domain independent of the one required for E2F binding (42). p107 forms stoichiometric complexes with these kinases and E2F in a temporally defined manner, with the p107-cyclin E-cdk2 complex appearing in late G_1 phase and p107-cyclin A-cdk2 appearing later in S phase (3, 29, 37).

Biochemical and structural studies have identified an amino acid sequence in the spacer region of p107 required for binding cyclins (43), and related sequences have been found in other cyclin-binding proteins. This short sequence motif, termed the LFG motif for the residues important for the interaction, was initially identified in the p21-p27-p57 family of mammalian and *Drosophila* CKIs (9, 27), and structural studies have established the importance of this motif in p27-cyclin A interactions (33). A similar sequence was noted in the spacer region of p130, and a related, but nonidentical, sequence was identified in the E2F family of transcription factors (1, 25, 26). Interestingly, this E2F sequence is necessary and sufficient for conferring cyclin A-cdk2 binding to certain members (E2F-1, -2, and -3) of this transcription factor family but not others, resulting in their phosphorylation and inhibition of activity (14).

Previously, we showed that in vitro reconstitution of stoichiometric complexes containing either p107 or p130 and cyclin A-cdk2 or cyclin E-cdk2 resulted in the loss of kinase activity directed toward an exogenous substrate, histone H1 (41). Interestingly, endogenous p130-kinase complexes isolated from human cells exhibited similar properties, and we could distinguish two cellular p130-cyclin-cdk2 complexes that lacked and contained associated E2F activity.

In this study, we have begun to address the mechanism by which p107 regulates the activity of associated cdk2 in vivo and in vitro. We have surveyed cells lacking p107 and the related p130 protein and found that the total kinase activity associated with cdk2 increases in these cells, and in complementary experiments modest increases in p107 expression in human cells significantly decreased endogenous cdk2 activity. By several biochemical criteria, we show that p107 can act as a bona fide CKI with an inhibitory constant (K_i) similar to that of p21/WAF1. Although p107 is a strong substrate for cyclin A-cdk2, cyclin D-cdk4, and cyclin E-cdk2, the ability to dissect regions of the protein that function as efficient substrates but not

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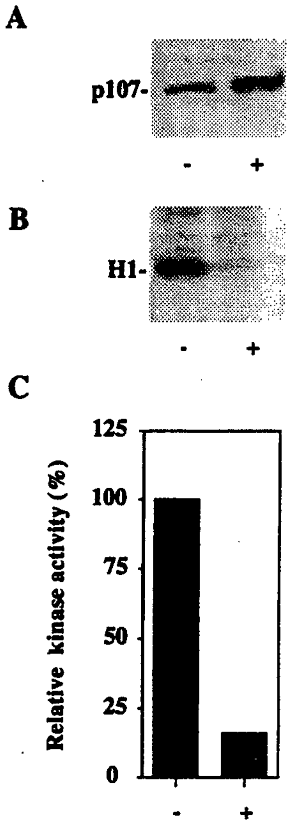


FIG. 1. p107 and p130 are inhibitors of cdk2 activity in vivo. (A) p107 expression is modestly induced in a tetracycline-repressible cell line by removal of tetracycline. +, p107 induction; −, expression was not induced. (B) Induction of p107 levels shown in panel A causes significant reduction in histone H1 kinase activity. Results of a representative experiment are shown. (C) Quantitation of kinase activity shown in panel B.

inhibitors suggests that inhibition does not occur simply by a preferred-substrate mechanism. In distinguishing between cyclin-cdk2 substrates and inhibitors, our experiments also point to a major difference between p107 and its relative pRB: while the former is an effective inhibitor in vitro, the latter is not. Through systematic mutagenesis of p107, we define a previously uncharacterized portion of p107 that can inhibit both cyclin A-cdk2 and cyclin E-cdk2. Interestingly, this region of p107 contains a sequence related to other cyclin-binding domains, and we show that in some settings, it is required in vivo for growth suppression. These findings prompt a comparison with the CKI p21, which also inhibits cdk activity through dual cyclin-binding sites.

MATERIALS AND METHODS

Cell lines and transfections. The human osteosarcoma cell line Saos-2 and cervical carcinoma C33A were obtained from E. Harlow. Transfections, nocodazole treatment, and fluorescence-activated cell sorter (FACS) analysis were performed essentially as described previously (42). Briefly, for C33A transfections, cells were transfected with 2 μg of cytomegalovirus (CMV)-CD20 and 23 μg of each p107 or p130 expression plasmid (except for the p130AA and p130AAA mutants, in which case 11.5 μg of DNA was used), unless noted otherwise, by standard calcium phosphate methods. After 12 to 14 h, precipitates were removed and cells were washed twice with phosphate-buffered saline and allowed to incubate further for 24 h. Cells were then treated with 40 ng of nocodazole per ml for an additional 12 h and harvested for FACS and Western blot analyses. CD20-positive cells were analyzed with a FACScan (Becton Dickinson) equipped with CellQuest and ModFit software. Data are presented as averages from at least three separate transfection experiments.

Plasmids and peptides. Deletion and point mutageneses were carried out by PCR. Construction of the p107ΔSA mutant involved digestion of CMV-p107 with *Sph*I and *Acc*I, creation of blunt ends with T4 polymerase, and ligation. To create an expression vector for Δ10N protein containing the first 409 amino acids of p107, a *Bam*HI-*Dra*III fragment was excised from CMV-p107Δ10, T4 DNA polymerase blunted, and subcloned into *Bam*HI-*Eco*RI-digested, Klenow polymerase-blunted pGEX-KG. Δ10NAAA was made by replacing an *Eag*I-*Bsm*I fragment from pGEX-KG-Δ10N with the corresponding one from CMV-p107AAA. All mutations were confirmed by DNA sequencing. Oligonucleotide sequence information and details for plasmid construction are available upon request. The glutathione *S*-transferase (GST)–p53 expression plasmid was a gift of H. Lu.

The following p107 peptides were synthesized: p107N (ACRKSIIPTV), p107N-mut (AAAASIIPTV), and p107S (SAKRRLFGED). All peptides were synthesized by Research Genetics, Inc.

Antibodies. Polyclonal antibodies against p107 (C-18), cyclin A (H-432), cdk2 (M2), and p130 (C-20) were obtained from Santa Cruz Biotechnology, and anti-flu hemagglutinin (HA) antibody 12CA5 was obtained from Berkeley Antibody Co. Monoclonal antibodies against cyclin E (HE12) and p107 (a mixture of SD2, -4, -6, and -9 used for immunoprecipitations or SD9 used for Western blotting) were provided by N. Dyson and E. Harlow. For immunoprecipitations that were followed by Western blotting, antibodies were first coupled to protein A-Sepharose by standard methods (20).

Recombinant protein production. p107, N385, and pRB were purified to near homogeneity by chromatography over an affinity column bearing amino acid residues 20 to 29 of human papillomavirus E7 as described previously (13) except that the column was washed with an additional step of 1.0 HMGNB (25 mM HEPES [pH 7.6], 1 M NaCl, 10% glycerol, 0.1% Nonidet P-40 [NP-40], 5 mM β-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride [PMSF]). Eluates from the E7 column were subsequently dialyzed against 0.1 HEGNDP buffer (100 mM KCl, 25 mM HEPES [pH 7.6], 0.1 mM EDTA, 10 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol [DTT], 0.01% NP-40, and 0.2 mM PMSF). In experiments involving cyclin D-cdk4, buffer D (150 mM NaCl, 50 mM HEPES [pH 7.6], 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1% Tween 20, 5 mM β-mercaptoethanol, and 0.2 mM PMSF) was used instead of 0.1 HMGNB. p21 was produced in bacteria and purified as described previously (43).

Purification of GST-tagged proteins. GST-tagged recombinant Δ10N and p53 were overproduced and purified as follows. After a 1-h induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C, bacteria were pelleted, washed once in phosphate-buffered saline, and sonicated in 0.1 HEMGDP buffer containing 5 μg of leupeptin per ml and 5 μg of aprotinin per ml. Following sonication, lysates were precleared by centrifugation, and the resulting supernatants were incubated with glutathione-agarose for 1 h at 4°C. After extensive washing with 0.1 HEMGDP, the proteins were eluted with elution buffer (100 mM Tris [pH 7.9], 120 mM NaCl, 7 mg of glutathione per ml, 1 mM DTT, and 0.2 mM PMSF). Proteins eluted from the glutathione-agarose beads were subsequently dialyzed against 0.1 HEMGDP. GST-cyclin A-cdk2, GST-cyclin E-cdk2, and GST-cyclin D-cdk4 complexes were produced in insect cells by previously described methods (13, 14, 41). These complexes were purified as for all other GST-tagged proteins. Baculoviruses encoding HA-tagged cdk2 and GST-cyclins were generously provided by D. Morgan, W. Harper, and H. Piwinica-Worms.

In vitro kinase assays. For kinase inhibition assays, purified p107, Δ10N, Δ10N-AAA, N385, or p21 was preincubated at room temperature for 30 min in kinase buffer (50 mM HEPES [pH 7], 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 0.2 mg of bovine serum albumin per ml, 10 mM NaF, and 0.2 mM PMSF) with ~0.8 ng of purified cyclin A-cdk2 or cyclin E-cdk2. Where noted, 1 μM ATP was used to phosphorylate p107 during the preincubation. Kinase buffer with 1 μM ATP, 2.5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol), and 100 ng of the indicated substrate (or 1.25 μg in the case of histone H1) was then added, and the reactions were allowed to proceed for 15 min at 37°C. For kinase assays involving peptide competition, reactions were carried out by adding the indicated peptide at 15 μM during the preincubation. Phosphorylation levels were quantified with a PhosphorImager, and the data were plotted as percentages, with 100% representing the value for the reactions without any inhibitor. The values reported represent the averages and standard errors for three independent experiments.

Peptide competition assays. Peptide competition assays were carried out by incubation of 40 ng of p107 with 40 ng of cyclin A-cdk2 or cyclin E-cdk2 for 1 h at 4°C in 20 μl of kinase buffer in the presence or absence of the indicated amount of peptide followed by a 1-h incubation with 500 μl of 0.1 HEGNDP and 5 μl of a 50% slurry of glutathione-agarose beads. After extensive washing with 0.1 HEMGDP, the precipitated proteins were then resolved on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and visualized by Western blot analysis as follows. After electrophoresis, the gels were transferred to a polyvinylidene difluoride membrane (Millipore), and the membranes were blocked for 1 h with 5% nonfat milk in Tris-buffered saline at pH 8.0 with 0.2% Tween 20, washed, and incubated with anti-p107, anti-cyclin A, or anti-cyclin E antibodies. The blots were then washed and developed by using an enhanced chemiluminescence detection system (NEN).

p107 and pRB binding assays. Phosphorylation-binding experiments were carried out by incubation of 100 ng of p107 or 100 ng of pRB with 40 ng of cyclin A-cdk2 and 2.5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) for 30 min at 37°C followed by a 1-h incubation with 500 μl of 0.1 HEGNDP and 5 μl of a 50% slurry of glutathione-agarose beads. After extensive washing with 0.1 HEGNDP, the

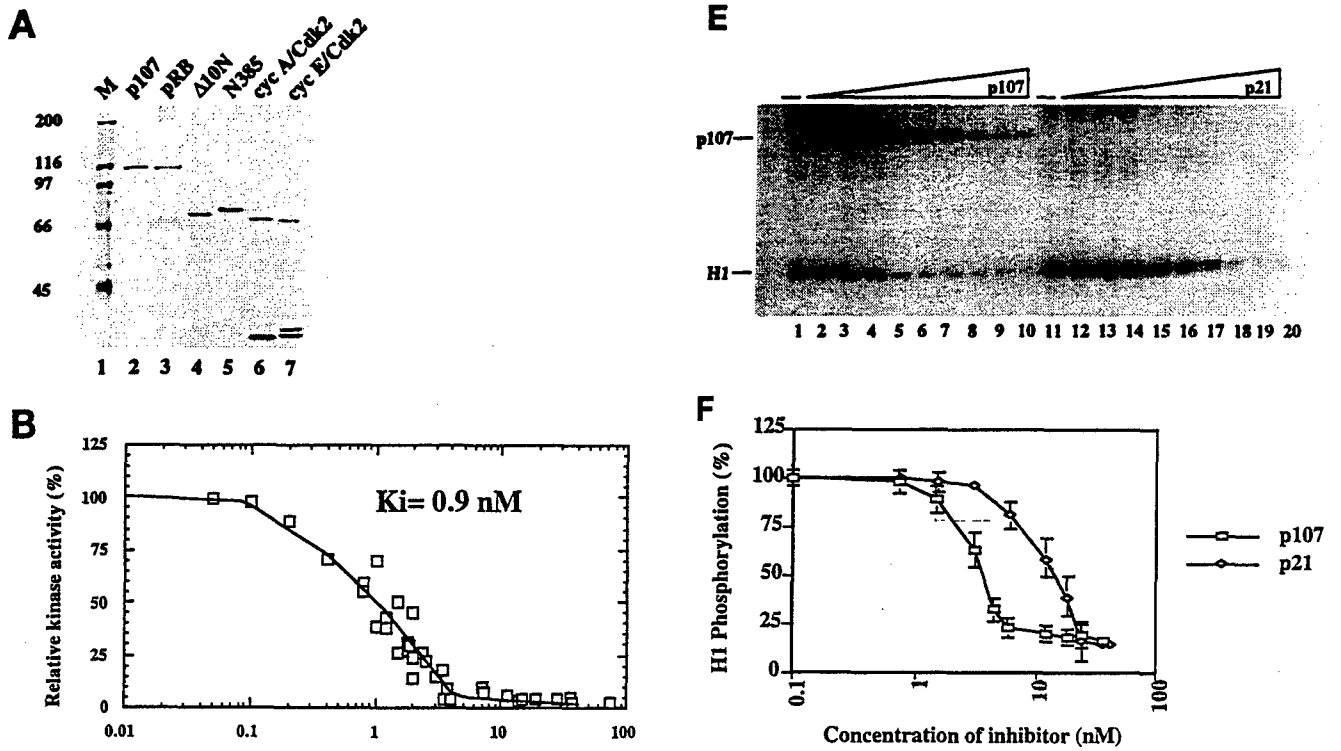


FIG. 2. Inhibition of cyclin A (cyc A)- and cyclin E-cdk2 by p107 and p21 in vitro. (A) Recombinant proteins used in the in vitro assays. p107, pRB, N385, cyclin A-cdk2, and cyclin E-cdk2 were purified from insect cells infected with recombinant baculoviruses. GST-tagged Δ10N was purified from bacteria as described in Materials and Methods. Equal amounts of the indicated proteins were electrophoresed and visualized by silver staining. The sizes of molecular weight markers (lane M) (in thousands) are indicated on the left. (B to D) Histone H1 kinase assays were performed with purified p21 from *Escherichia coli* or purified p107 (see Materials and Methods). Increasing concentrations of p21 (0.05 to 74 nM), p107 (0.01 to 30 nM), or prephosphorylated p107 (0.01 to 30 nM) were used as indicated. The relative levels of phosphorylated histone H1 were determined with a PhosphorImager. Histone H1 phosphorylation by cyclin A-cdk2 in the absence of an inhibitor was given a value of 100. Calculated average K_i values are indicated. (E) Histone H1 kinase assays comparing p21 and p107 as inhibitors of cyclin E-cdk2. Increasing concentrations of p107 (0.7 to 42 nM) (lanes 2 to 10) or p21 (0.7 to 42 nM) (lanes 12 to 20) were incubated with 1 ng of cyclin E-cdk2. For each set of reactions, a kinase-alone control (lanes −) was included. (F) The relative levels of phosphorylated histone H1 were determined as for panels B to D. Each value represents the mean and standard error of the mean for four independent experiments.

beads were resuspended in loading dye, boiled, loaded on an SDS–10% polyacrylamide gel, and visualized by autoradiography. Coprecipitation of cyclin A by p107 or Δ10N was carried out by incubating 50 ng of thrombin-cleaved GST-cyclin A or GST-cyclin E (thrombin was used to remove the GST moiety) with 100 ng of p107, N385, Δ10N, or Δ10N-AAA for 1 h at 4°C. Complexes were collected either by GST precipitation as described above or, in the case of p107 and N385, with 5 μl of E7 beads substituted for the GST beads.

Immunoprecipitations and deoxycholate release. Extracts were generally made by cell lysis on ice for 30 min in E1A lysis buffer (50 mM HEPES [pH 7], 5 mM EDTA, 250 mM NaCl, 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 10 mM NaF, and 50 mM β-glycerophosphate). For the analysis of p107 and p130 expression and cyclin association, approximately 25 to 200 μg of total protein was immunoprecipitated. Release of E2F associated with p107 by deoxycholate treatment and E2F gel mobility shift assays have been described previously (42).

The tetracycline-repressible p107 cell line (44) (a gift of L. Zhu and E. Harlow) was grown in Dulbecco modified Eagle medium containing 10% fetal calf serum, 500 μg of G418 per ml, and 1 μg of tetracycline per ml. After the cells were grown to 70% confluence, p107 was induced by removal of tetracycline. The cells were grown for 48 h after removal of tetracycline, and extracts of these cells were compared with those obtained from cells that were grown in the presence of tetracycline. The extracts were made in E1 lysis buffer as described above, and 300 μg of total protein was used for the immunoprecipitation with anti-cdk2 antibody as described above. Immunoprecipitates were incubated in kinase reactions as described above.

RESULTS

p107 and p130 are physiological inhibitors of cdk2 activity. To test directly the notion that p107 is a physiological inhibitor of cdk2, we induced small increases in p107 expression in

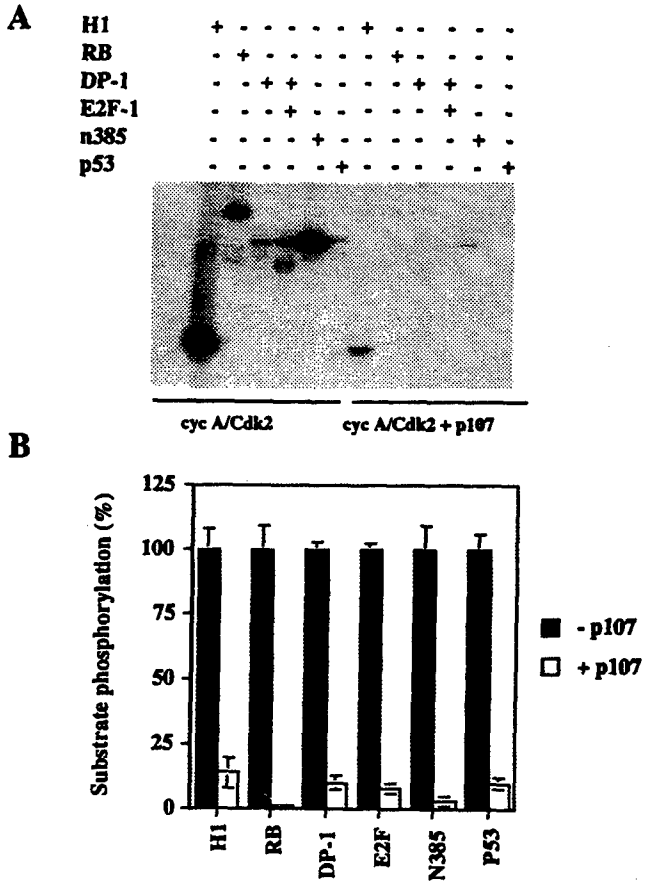


FIG. 3. Inhibition of cyclin A (cyc A)-cdk2 substrates. (A) Kinase assays were performed in the absence (left) or presence (right) of 20 ng of p107 (previously phosphorylated). One hundred nanograms of the indicated purified proteins was added to the reaction mixture, with the exception of histone H1, for which 1.25 μg was used. (B) The amount of phosphorylation of each protein was quantified with a PhosphorImager, and the level of phosphorylation of each protein in the absence of p107 was set to 100%. The values represent the means and standard errors of the means for four different experiments.

human Saos-2 osteosarcoma cells. In these cells, expression of p107 is controlled in a tetracycline-repressible manner (18, 44) (Fig. 1A). Here, removal of tetracycline led to a modest increase in p107 levels with a concomitant significant decrease in cdk2 kinase activity (Fig. 1B and C). These differences could not be ascribed to changes in the amounts of cyclin A, cyclin E, or cdk2 protein, since their levels were not altered upon induction of p107 (data not shown). Thus, we conclude that p107 is an inhibitor of cdk2 in vivo.

In further experiments, when we immunoprecipitated cdk2 from lysates derived from wild-type, $p107^{-/-}$, and $p107^{-/-}$ $p130^{-/-}$ mouse embryo fibroblasts (MEFs) (6, 24, 28), we noticed that cdk2-associated histone H1 kinase activity was elevated in either mutant cell type relative to that in wild-type cells. We reproducibly observed a 2-fold increase in kinase activity in $p107^{-/-}$ cells and a 2- to 2.5-fold increase in $p107^{-/-}$ $p130^{-/-}$ cells (data not shown). While these differences are modest, they are in keeping with the fact that the mutant cells have retained a host of biochemically redundant CKIs, namely, p21/WAF1, p27, and p57.

Unphosphorylated p107 and phosphorylated p107 inhibit cdks with an apparent K_i similar to that of p21/WAF1. Previously we had shown that stoichiometric cyclin-cdk2 complexes containing either p107 or p130 exhibited greatly diminished kinase activity toward an exogenous substrate, histone H1, relative to free kinase complexes (41). However, given that both pRB-related proteins bound tightly to cyclin A-cdk2 and cyclin E-cdk2 and were potent substrates of these kinases, we were unable to distinguish between two potential mechanisms for kinase inhibition. In one scenario, p107 and p130 could inhibit each kinase in a manner similar to that of the p21-p27-p57 family of CKIs. Alternatively, p107 and p130 could act as preferred substrates to inhibit phosphorylation of exogenous substrates by simple substrate competition. Although the experiments described above render this latter possibility unlikely, we designed experiments using highly purified recombinant proteins (Fig. 2A) to test each possibility.

First, we compared the apparent inhibition constants (K_is) for cyclin A-cdk2 and cyclin E-cdk2 by using p107 and p21/WAF1, a known CKI. We performed histone H1 kinase assays in which we titrated increasing amounts of each inhibitor into the reaction mixtures. Remarkably, p107 and p21 exhibited similar apparent K_is for inhibition of cyclin A-cdk2, and we calculated K_is of 1.3 and 0.9 nM for p107 and p21, respectively (Fig. 2B to D). This estimate for the inhibitory constant for p21 is in agreement with ones made previously (0.5 nM [21]). However, p107 and p21 were markedly less potent as inhibitors of cyclin E-cdk2 (Fig. 2E and F). Moreover, p107 and p21 differentially inhibited cyclin E-cdk2, with K_is of 3.3 and 13.6 nM, respectively. This estimate for inhibition of cyclin E-cdk2 by p21 differs somewhat from the previously calculated value (3.7 nM [21]), perhaps due to differences in reaction conditions. We have also noted that purified, recombinant cyclin D-cdk4 robustly phosphorylates p107, consistent with a previous report (2), but cyclin D-cdk4 kinase activity is not significantly affected by amounts of p107 that abolish cyclin A-cdk2 activity (data not shown), suggesting that kinase inhibition by p107 is specific for cdk2-associated kinases.

As expected, p107 was phosphorylated during the course of the kinase reaction. In principle, then, enhanced p107 phosphorylation could account for the decreased phosphorylation of histone H1. To rule out this possibility, we determined whether phosphorylated p107 was equally capable of inhibiting the phosphorylation of histone H1 by cyclin A-cdk2. Here, p107 was phosphorylated with unlabeled ATP and subsequently incubated with labeled ATP and substrate. In this experiment, further phosphorylation of p107 was not apparent, attesting to the fact that prephosphorylation of p107 approached completion (data not shown). Interestingly, phosphorylated p107 had a K_i similar to that of unphosphorylated p107 and p21 (Fig. 2B to D). The results of this experiment (and those presented below) argue further against a substrate competition model for kinase inhibition by p107 and instead support the idea that p107 is a direct and potent inhibitor of cyclin-cdk2 complexes. Since phosphorylated p107 retained the ability to inhibit cyclin A-cdk2, we determined whether the phosphorylated protein would be capable of stable binding to the kinase. Both forms of p107 bound cyclin A-cdk2 to similar extents (see Fig. 4A and B), in agreement with their comparable K_i values.

p107 inhibits phosphorylation of several cyclin A-cdk2 substrates. Earlier experiments did not address the possibility that p107 could preferentially inhibit the phosphorylation of some substrates but not others, in effect redirecting substrate usage by cdks. We reasoned that if p107 was truly an inhibitor of cyclin-cdk2 complexes, phosphorylation of all substrates should be diminished in the presence of this protein. We therefore tested the phosphorylation of several known cyclin A-cdk2 substrates. Only a handful of substrates have been identified thus far, and these include pRB, E2F-1 and its heterodimeric

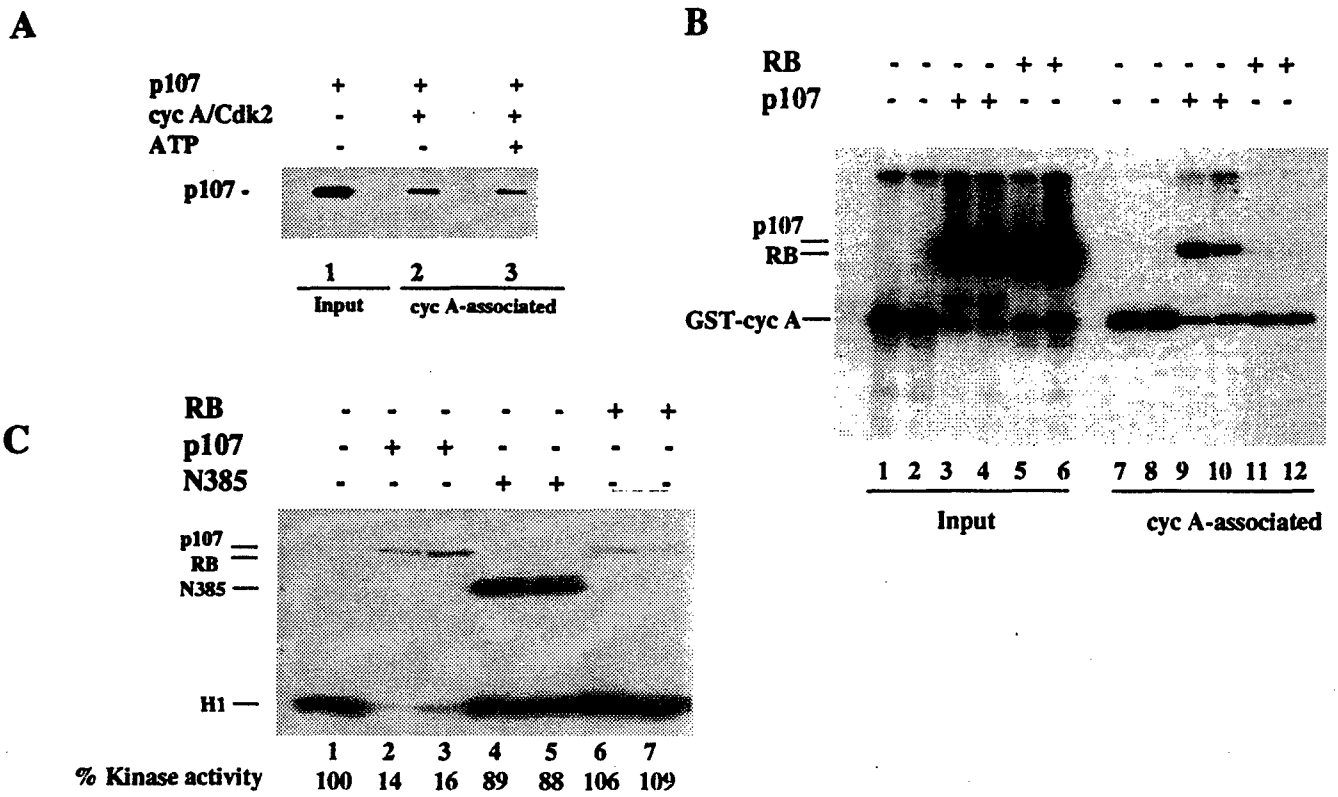


FIG. 4. p107 and pRB as inhibitor and substrate, respectively, of cyclin A (cyc A)-cdk2. (A) p107 was allowed to bind cyclin A-cdk2 in the absence or presence of 1 μM ATP for 30 min at 37°C. Glutathione-agarose precipitation of GST-cyclin A-cdk2 was carried out as described in Materials and Methods, followed by immunoblot analysis of p107. The input amount of p107 is shown in lane 1. (B) p107 (lanes 3, 4, 9, and 10) and pRB (lanes 5, 6, 11, and 12) were incubated in a kinase reaction without histone H1 and with [γ-^{32}P]ATP. After 30 min at 37°C, half of the reaction mixture (lanes 1 to 6) was taken and boiled in SDS sample buffer, while the other half (lanes 7 to 12) was incubated with glutathione-agarose beads and precipitated as described in Materials and Methods. (C) Cyclin A-cdk2 kinase reactions in the presence of 20 ng of p107 (lanes 2 and 3), 60 ng of N385 (lanes 4 and 5), or 20 ng of pRB (lanes 6 and 7). (D) An extended titration comparing the effects of equal amounts of pRB and p107 on cyclin A-cdk2 kinase activity. The amount of either protein is indicated below the autoradiogram. (E) Quantitation of the data shown in panel D. The relative percentage of kinase activity was determined in each case by the amount of ^{32}P incorporated into histone H1, as measured with a PhosphorImager.

partner DP-1, and p53 (reviewed in reference 12). When equivalent amounts of these proteins (as judged by silver staining) were added to kinase reaction mixtures, phosphorylation of each substrate was considerably reduced, and in every case, the level of inhibition was also comparable (Fig. 3).

pRB does not inhibit cyclin A-cdk2. The p107 protein displays significant similarity with pRB, particularly in the carboxy-terminal half of the protein. To determine whether pRB was likewise able to inhibit cyclin A-cdk2, we performed comparisons between pRB and p107 under identical assay conditions. Although pRB is phosphorylated by the kinase to the same degree as p107, unlike p107, it cannot stably bind cyclin A-cdk2 after phosphorylation (Fig. 4B). More importantly, when we titrated equal amounts of p107 and pRB into kinase reaction mixtures (Fig. 4C) containing cyclin A-cdk2, we noted a striking difference in histone H1 phosphorylation. Here, p107, but not pRB, was able to inhibit cyclin A-cdk2. In further titration experiments (Fig. 4D and E), even a fivefold excess of pRB (relative to an amount of p107 that produced complete

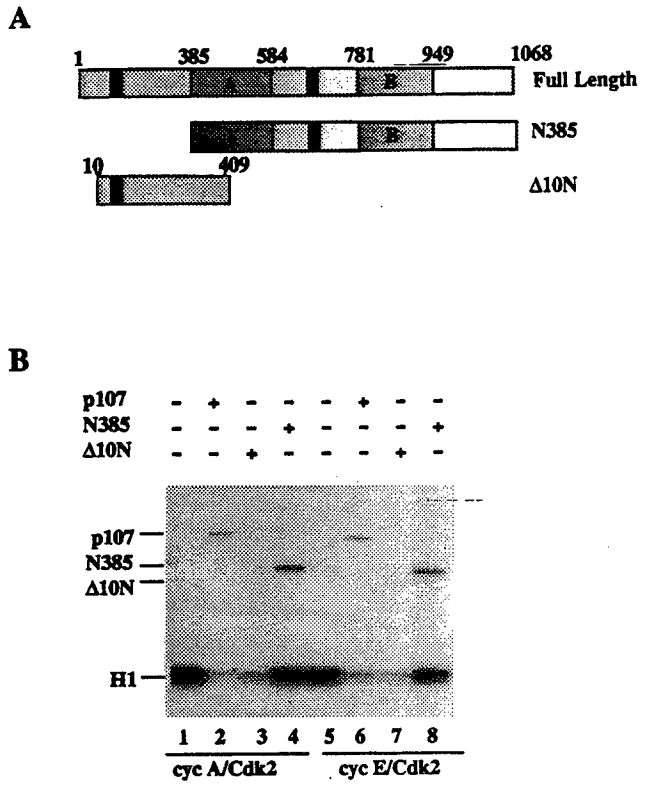


FIG. 5. Inhibition of cyclin A (cyc A)- and cyclin E-cdk2 by the amino terminus of p107. (A) Schematic diagram of p107, N385, and Δ10N proteins. Shaded rectangles with A and B denote subdomains of the carboxy-terminal region conserved with pRB and p130, and black rectangles indicate potential amino-terminal and spacer region cyclin-binding domains. (B) Kinase reaction comparing equal amounts (approximately 20 ng) of p107 (lanes 2 and 6), Δ10N (lanes 3 and 7), and N385 (lanes 4 and 8). All of the reaction mixtures contained 1.25 μg of histone H1 as a substrate for cyclin A-cdk2 (lanes 1 to 4) or cyclin E-cdk2 (lanes 5 to 8), and each had been preincubated at room temperature in the presence of unlabeled 1 μM ATP. The positions of phosphorylated histone H1 and p107 proteins are indicated at the left.

inhibition) did not significantly inhibit this kinase (80% of histone H1 kinase activity was retained). pRB was nevertheless heavily phosphorylated in these experiments (Fig. 4D). We conclude that pRB is a potent substrate for, but not an inhibitor of, cyclin A-cdk2.

An amino-terminal domain of p107 inhibits cdks. In earlier work, we had demonstrated that p107 required an amino-terminal domain to inhibit associated cyclin-cdk2 complexes completely (41). Consistent with this observation, when we titrated a purified carboxy-terminal fragment of p107 lacking 385 amino-terminal residues (termed N385) into kinase reaction mixtures, phosphorylation of histone H1 was not significantly altered, although the N385 protein was an excellent substrate (Fig. 4C, compare lanes 1 to 5). Notably, when equal amounts of protein were compared, N385 was phosphorylated to a greater extent than full-length p107 (data not shown and see Fig. 10E), confirming the idea that an inhibitory domain had been removed.

Since our experiments had identified a region in the amino-terminal one-third of p107 important for kinase inhibition, we examined the ability of a purified amino-terminal fragment to inhibit both cyclin A-cdk2 and cyclin E-cdk2. Figure 5A shows a representation of several p107 proteins that were tested. Interestingly, the GST-tagged amino-terminal fragment (called Δ10N) was a potent inhibitor of both kinases, although complete inhibition required a fivefold molar excess relative to full-length p107 (Fig. 5B). As a control, we could show that excessive (10-fold-larger) amounts of purified GST protein lacking additional residues had no effect on kinase reactions (data not shown). In contrast with the N385 protein, Δ10N is not significantly phosphorylated (data not shown and see Fig. 10E). Thus, we have identified both a carboxy-terminal region in p107 (N385) that is a potent substrate of cdks but which does not inhibit kinase activity and an amino-terminal fragment that is independently able to inhibit cyclin A-cdk2.

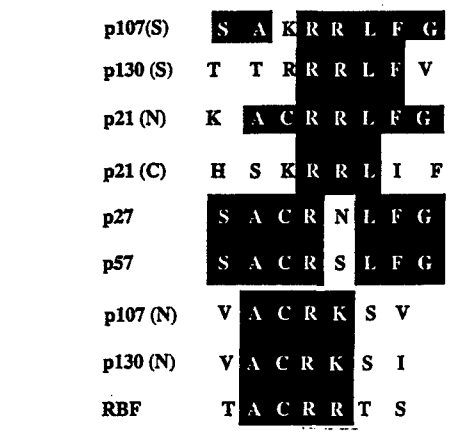
The amino-terminal region of p107 is critical for growth suppression of C33A cells. Previous studies had indicated that residues within the first 110 amino acids of p107 were important for growth suppression and complete inhibition of associated kinases (41). These data and those described above led us to examine this region of p107 and p130 in greater detail to delineate the location of a region potentially important for both cyclin binding and growth suppression. In parallel studies, we tested the effects of deletions and point mutations on cyclin binding and kinase inhibition.

Recent biochemical and structural studies have identified a novel motif that confers tight binding to cyclins (Fig. 6A). This LFG motif is critical for interactions with several families of proteins, including the p21-p27-p57 group of CKIs, E2Fs, and the spacer regions of p107 and p130 (1, 5, 31, 33, 43). Interestingly, a second region with limited homology to the LFG motif was identified near the amino terminus of p107 and spanned residues 66 to 69 (Fig. 6A). Although this region of the protein is not highly conserved in pRB, it is strongly conserved in p130 and the *Drosophila* pRB-related factor, RBF (11, 19, 30, 45).

To begin examining the relevance of this region to cyclin binding, we took advantage of a growth suppression assay in which C33A human cervical carcinoma cells were cotransfected with various p107 test plasmids and a plasmid expressing the CD20 cell surface marker (45). CD20-positive cells were scored for cell cycle distribution by flow cytometry. To define a region important for growth suppression, we initially produced p107 deletions that removed 10, 24, 38, 76, and 110 amino-terminal residues, as well as an internal deletion of residues 68 to 132 (p107ΔSA) (Fig. 6B). Although we succeeded in expressing three deletions, p107Δ10, p107Δ110, and N385, we were unable to express many of these proteins at levels equivalent to that of full-length p107 owing to their instability in cells (data not shown).

As expected, expression of p107 provoked a potent G_1 arrest, as evidenced by a large increase in this population, while the mutants truncated by 110 and 385 amino-terminal residues had little effect on cell cycle progression (Fig. 6B and C). On the other hand, p107Δ10 was nearly as potent a growth suppressor as the wild-type protein. This suggested that the region of interest is located between residues 10 and 110. Furthermore, since the protein encoded by p107ΔSA was unable to prevent cell proliferation, residues between 68 and 110 were clearly important for growth suppression. We also transfected a p107 construct termed L19 that lacks an E2F-binding domain but which retains the ability to suppress cell growth (Fig. 6A) (42). Starting with L19, we derived additional, stable amino-terminal deletion mutants of L19 that behaved in a manner similar to that of the corresponding mutants able to bind E2F described above (Fig. 6C).

To rule out the possibility that the effects described above were specific to C33A cells, we also transfected the human osteosarcoma cell line Saos-2 with L19 and mutant derivatives thereof. Use of the p107 L19 mutant bypasses growth suppres-



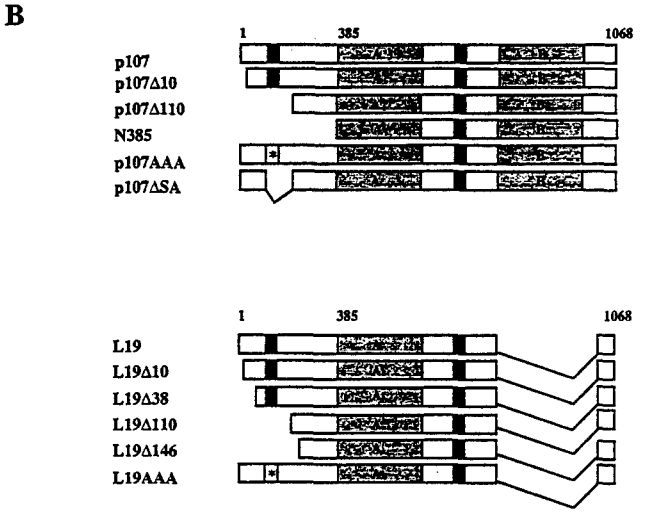
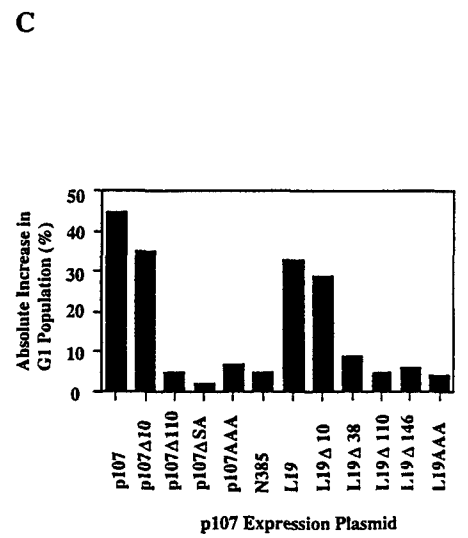
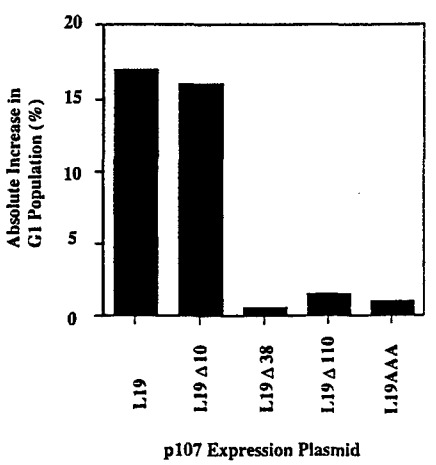


FIG. 6. An amino-terminal region in p107 is important for growth suppression. (A) Schematic alignment of cyclin-binding domains found in the p21-p27-p57 and p107-p130 families of proteins. Previously identified sequences found in the spacer regions (S) of p107 and p130 are indicated. Homologous amino-terminal sequences (N) found in p107, p130, and the *Drosophila* pRB-related factor, RBF, are shown. (B) Diagram of p107 constructs used in transfection assays. Symbols used are as indicated in the legend to Fig. 5. Deletions and triple alanine point mutations (asterisks) are indicated. (C) Growth arrest of C33A cells mediated by p107 as determined by FACS analysis (see Materials and Methods). The ordinate shows the absolute difference in percentage of cells in the G_1 phase of the cell cycle. Values represent averages for at least three independent experiments. (D) Growth arrest of Saos-2 cells by p107 L19 derivatives.





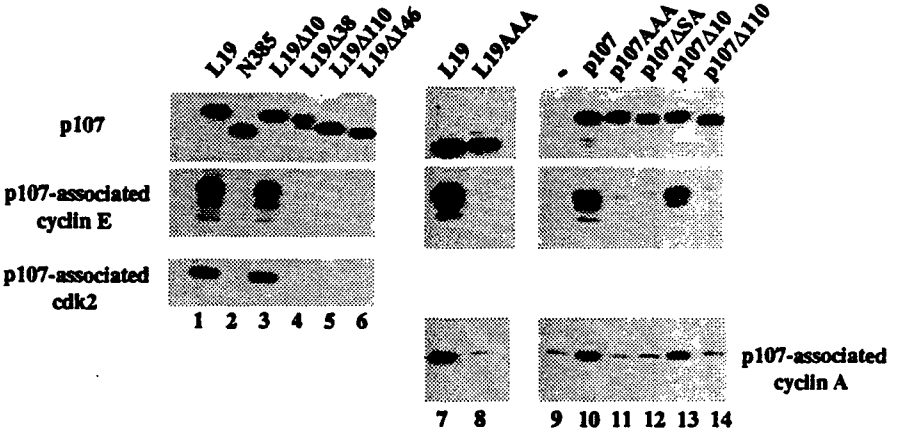


FIG. 7. Mutations in p107 abolish binding to cyclins A and E. Each of the indicated proteins was expressed in C33A cells by transfection, and whole-cell extracts were immunoprecipitated with antibodies against p107 or the HA tag (which recognize the carboxy-terminal tag on p107 constructs). Immunoprecipitates were electrophoresed and subjected to immunoblotting with the indicated antibodies. A negative control transfection with empty expression vector (lane −) is indicated.

sion through E2F that occurs in this, but not the C33A, cell line (42). As shown in Fig. 6D, mutation of a putative amino-terminal domain dramatically compromised the growth-suppressive properties of p107 to an extent similar to that seen with C33A cells.

Identification of a second region in p107 important for cyclin binding. Having identified a functionally important region between residues 10 and 110 that contains sequence similarity to other cyclin interaction sites, we made several additional deletions that specifically target this region of p107. Significantly, one p107 mutant, p107ΔSA, has two residues (arginine and lysine) of the putative amino-terminal cyclin-binding motif deleted and is unable to suppress growth of C33A cells (Fig. 6A and C). To confirm the idea that this region was critical for growth suppression, we generated clustered point mutations in p107 that converted the cysteine, arginine, and lysine residues to alanines (mutant p107AAA). Although expressed at levels equivalent to that of the wild-type protein, this mutant was unable to suppress growth of C33A cells (Fig. 6C). Likewise, the corresponding L19 mutant (L19AAA) was completely impaired in its ability to arrest both C33A and Saos-2 cells (Fig. 6C and 6D).

Next, we tested the ability of each p107 mutant to bind endogenous cyclins after expression in C33A or Saos-2 cells. As shown in Fig. 7, antibodies recognizing either p107 or a flu HA tag on L19, or the Δ10 derivative of each, coprecipitated abundant amounts of cyclin A, cyclin E, and cdk2 (lanes 1, 3, 7, 10, and 13). In marked contrast, each of the larger amino-terminal deletions and the p107ΔSA deletion completely eliminated cyclin binding. Notably, the p107AAA and L19AAA mutants no longer associated with either cyclin (lanes 8 and 11). Thus, there was a direct correlation between the abilities of p107 to bind cyclins and suppress cell growth. Immunoblotting of identical samples of cell extracts indicated that the overall levels of cyclins A and E had not been altered by overexpression of p107 derivatives (data not shown). Although the deletion of 38 amino-terminal residues unexpectedly abrogated both cyclin binding and growth suppression (Fig. 6C and D and 7), we surmise that this indirectly results from the proximity of the deletion to the cyclin-binding region, which could destabilize its ability to bind cyclins. Alternatively, additional residues amino terminal to the putative cyclin-binding region could be important in stabilizing the association between cyclins and p107.

p130 mutants lacking the putative cyclin-binding domain are partially compromised in their growth-suppressive activity. We also tested the effect of mutations of the ACRK region of p130 on growth suppression by using assays identical to those described for p107. Using sequence alignments, we produced deletion mutants of p130 that were similar to those generated for p107. These deletions removed 16, 66, and 138 amino-terminal residues and have deletion endpoints that correspond to the p107 Δ10, Δ38, and Δ110 mutants (Fig. 8A). In addition, we constructed clustered point mutations of the ACRK region of p130, converting the cysteine and arginine residues to alanines (p130AA mutant) or converting all three residues to alanine (p130AAA, which corresponds to the p107AAA mutant). We observed comparable levels of expression of p130 and each of the p130AA and p130AAA mutants and robust expression of the deletion derivatives (Fig. 8B). Each of these proteins was then immunoprecipitated from C33A cells after transfection, and coprecipitation of cyclin E and cyclin A was tested by immunoblotting. The pattern of cyclin association with each of the mutants recapitulated that seen with the corresponding p107 mutant (Fig. 7). Significantly, mutation of either the cysteine and arginine or all three residues resulted in a level of cyclin binding comparable to that of the negative control lacking exogenous p130 expression (Fig. 8B, compare lanes 1, 3, and 4).

Intriguingly, although the p130AA and p130AAA mutants had apparently lost the ability to bind cyclins in vivo and appeared to have reduced growth-suppressive activity, the extent of this defect differed from that of the corresponding p107AAA mutant (compare Fig. 8C with 6C). While the basis for this residual inhibitory activity is presently unknown, it is possible that the growth-suppressive properties of p130 could arise in part from additional mechanisms not utilized by p107.

Taking these observations and our observations on p107 together, we conclude that p107 and p130 require two binding sites for potent in vivo interactions with cyclins and growth suppression activity.

The mutant p107 and p130 proteins retain E2F-binding activity. Given the rather severe effects on cyclin binding caused by deleting the amino-terminal domains of p107 and p130, it was important to determine whether the mutations globally disrupted the structures of these proteins. Since both of these proteins are able to bind cellular E2F, we relied on an assay in which endogenous E2F activity associated with p107 or

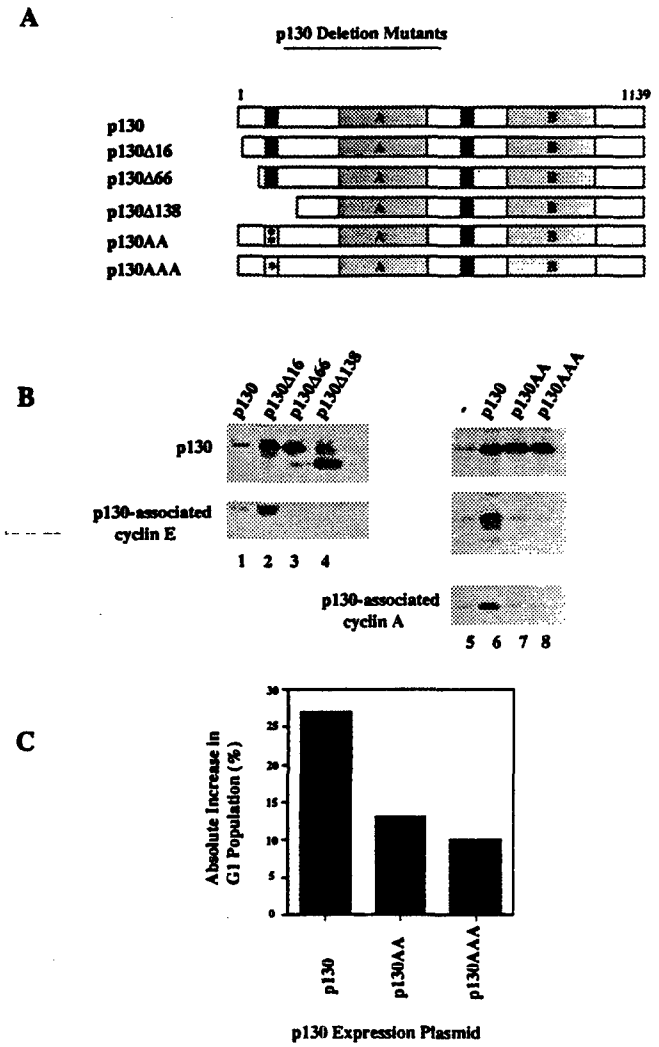


FIG. 8. Mutations in p130 similar to those introduced into p107 also abolish cyclin binding and diminish growth-suppressive activity. (A) Schematic of p130 mutations. Symbols are described in legend to Fig. 6. (B) Cyclin-binding activity of transfected p130 constructs. Endogenous cyclins were coimmunoprecipitated with p130 by using anti-p130 antibodies as described in the legend to Fig. 7. (C) Growth arrest of C33A cells as determined in Fig. 6C. The transfected p130 plasmids are indicated below the graph, and results represent averages for several independent experiments.

p130 is released by the detergent deoxycholate. E2F released by this treatment can be assayed for DNA-binding activity by using a gel mobility shift assay with a labeled oligonucleotide containing an E2F-binding site. Each of the amino-terminal mutants retained the ability to bind E2F (data not shown), suggesting that the loss of cyclin binding was not due to simple protein unfolding.

Mutations in the amino-terminal cyclin-binding domain abrogate kinase inhibition. Having determined that the amino-terminal region of p107 was important for growth suppression and cyclin binding in transfection experiments, we then tested the role of the amino-terminal cyclin-binding site in cyclin-cdk2 binding and inhibition in vitro. First, we tested the ability of the Δ10N protein to bind cyclin A and cyclin E. Purified cyclins interacted with both full-length p107 and Δ10N, although binding of Δ10N to both cyclins, especially cyclin E, was significantly reduced (Fig. 9A). In addition, the N385 protein (which contains the spacer region), like Δ10N, was able to bind

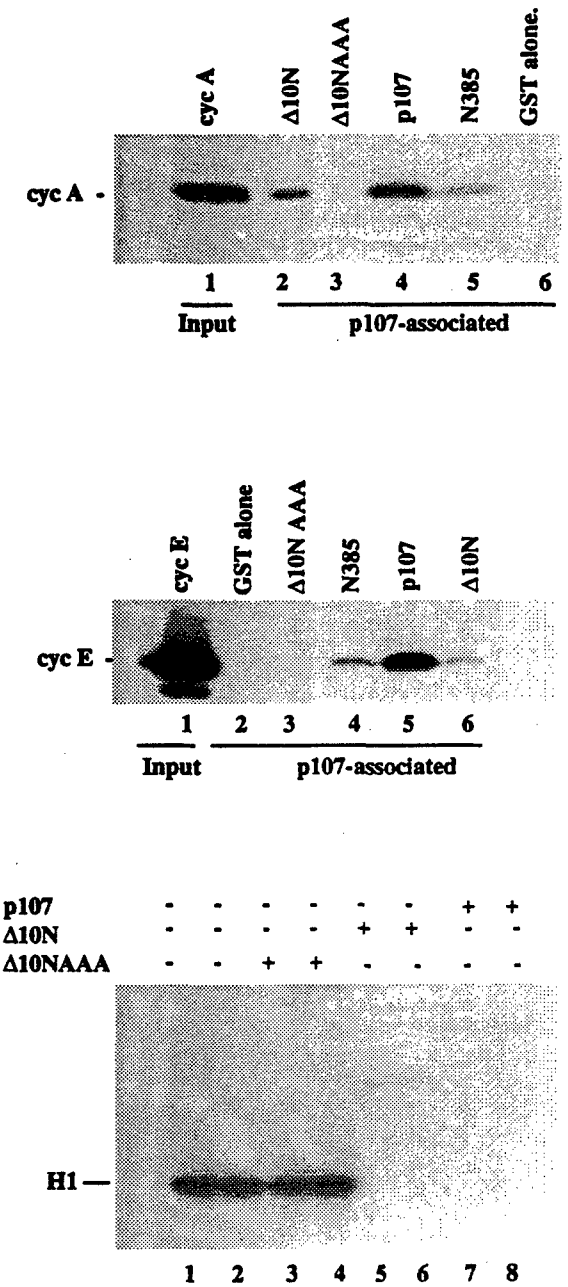


FIG. 9. Mutation of the p107 amino-terminal cyclin-binding domain abolishes kinase inhibition and binding. Cyclin A (cyc A) (A) and cyclin E (B) bind Δ10N and N385, albeit less efficiently than full-length p107, but do not bind the mutant Δ10N-AAA. (A) Western blot analysis of cyclin A bound to p107 derivatives. Thrombin-cleaved cyclin A, devoid of a GST tag, was tested for binding to GST alone (lane 2), Δ10N (lane 3), Δ10N-AAA (lane 4), p107 (lane 5), and N385 (lane 6). The input amount of cyclin A is shown in lane 1. (B) Western blot analysis of cyclin E bound to p107 derivatives. Thrombin-cleaved cyclin E, devoid of a GST tag, was tested for binding to GST alone (lane 2), Δ10NAAA (lane 3), N385 (lane 4), p107 (lane 5), and Δ10N (lane 6). The cyclin E input is indicated in lane 1. In each case, precipitation of N385 and p107 was performed with a human papillomavirus E7 peptide linked to Sepharose beads, and precipitation of Δ10N and Δ10N-AAA was performed with glutathione-agarose beads as described in Materials and Methods. (C) Kinase reactions comparing equal amounts of Δ10N-AAA (lanes 3 and 4), Δ10N (lanes 5 and 6), or p107 (lanes 7 and 8).

both cyclins, although it did so with lower efficiency than the full-length protein (Fig. 9A and B). Notably, however, only the amino-terminal Δ10N fragment was capable of inhibiting cdks. We conclude that p107 utilizes a second region near the amino terminus, in addition to the LFG domain residing in the spacer region, to bind and inhibit cyclin-cdk2 complexes.

We also constructed, purified, and tested a GST-tagged amino-terminal fragment identical to Δ10N (described above) in which the three alanine mutations present in p107AAA have replaced the wild-type residues. We titrated equal amounts of this protein, termed Δ10N-AAA, and Δ10N into kinase reaction mixtures containing cyclin A-cdk2. Δ10N and full-length p107 were potent inhibitors, as expected, while the mutant version of Δ10N failed to inhibit kinase activity (Fig. 9C, compare lanes 3 to 8). Moreover, relative to that with Δ10N, introduction of the triple alanine mutation severely impaired binding to both cyclin A and cyclin E (Fig. 9A and B).

Peptides spanning several cyclin-binding domains specifically compete p107 binding to cyclin-cdk2 and reverse inhibition of kinases by p107. As a final in vitro test of the specificity of interactions between cyclins and the amino-terminal cyclin-binding domain of p107, we performed competition experiments using peptides spanning the regions depicted in Fig. 6A as well as a peptide corresponding to the triple alanine point mutant of p107. Thus, peptides corresponding to the cyclin-binding motifs in the amino-terminal region of p107 (termed p107N) and the spacer region (termed p107S) were synthesized. In addition, a mutated peptide corresponding to p107N (p107N-mut) in which the cysteine, arginine, and lysine residues were converted to alanines was synthesized.

First, we determined whether coincubation of each peptide with p107 and cyclin A-cdk2 could effectively compete p107-kinase interactions. Figure 10 shows that peptide p107S effectively competed the binding of p107 to cyclin A-cdk2. Importantly, peptide p107N was also capable of competing this interaction, albeit more weakly, and the p107N-mut peptide was completely without effect at the highest concentration tested. As a control, we showed that the peptides did not interfere with retention of the cyclin A-cdk2 complex (lower panels in Fig. 10A to C). Experiments performed in parallel with cyclin E-cdk2 indicated that both the p107N and p107S peptides could compete the binding of this complex to p107, although each one was somewhat less effective at competing with p107 binding to this complex relative to the case for cyclin A-cdk2 (data not shown).

A similar profile was obtained when we titrated each of these peptides into kinase reaction mixtures in which cyclin A-cdk2 was inhibited by p107 (Fig. 10E). Both p107N and p107S were able to alleviate p107-mediated kinase inhibition (compare lanes 5 to 7). In contrast, the p107N-mut peptide did not alter inhibition by p107 at the highest concentration tested, nor did any of the peptides influence histone H1 kinase activity in the absence of p107 (Fig. 10E, lanes 1 to 4 and 8). Furthermore, we could show that peptides p107N and p107S, but not p107N-mut, could partially reverse kinase inhibition by the purified Δ10N fragment (lanes 8 to 12). As expected, the peptides had no effect on the activity of N385, which did not inhibit kinase activity (lanes 13 to 16). From these data, we conclude that the amino-terminal ACRK sequence found in p107 is important for mediating cyclin binding and kinase inhibition.

DISCUSSION

p107 was originally described as a pRB-related component of complexes that contained not only E2F but stoichiometric amounts of cyclin A-cdk2 and cyclin E-cdk2 as well (3, 15, 16, 37), and subsequent experiments suggested that p107 was a tightly binding substrate of these kinases. Our observations suggest that p107 not only is able to bind both kinases but also is capable of inhibiting the complexes to the same degree as most characterized CKIs (21). The inhibitory potential of p107 is not significantly affected by phosphorylation by the cyclin-cdk2 complex, not unlike certain CKIs, such as p27/Kip1, which are themselves phosphorylated during the process of inactivating these kinase complexes (35).

Furthermore, if p107 is a true kinase inhibitor, it should prevent the phosphorylation of known substrates. Indeed, this is the case. p107 can prevent the phosphorylation of several known cyclin A-cdk2 substrates, including pRB, E2F-1, DP-1, p53, and its own carboxy-terminal region, which contains most of the phosphorylation sites on p107, suggesting that p107 is capable of inhibiting the associated kinase rather than altering its substrate specificity, as suggested previously (22). In the previous study (22), it was shown that immunoprecipitates of p107 and p130 from cell extracts contained cyclin-cdk2 but showed little histone H1 kinase activity. However, the same immunoprecipitates nevertheless retained the ability to phosphorylate GST-pRB family protein fusions. Although the basis for this difference is currently unknown, it is important to note that our work has dealt with complexes reconstituted with highly purified proteins, while the work of Hauser et al. (22) has relied on immunoprecipitates of endogenous proteins, and the possibility that a contaminating kinase(s) activity was coprecipitated was not excluded.

Our results therefore suggest an important functional distinction between different members of the pRB family of proteins. All members of this family are thought to restrain cell growth as a consequence of transcriptional repression of E2F activity, and each is an excellent substrate for cyclin-cdk2 complexes. However, our work has distinguished at least two differences between pRB and p107 or p130. First, although pRB shows sequence similarity to p107 and p130, especially in the well-defined carboxy-terminal region (termed the pocket domain), it displays little similarity to these proteins in the amino-terminal region. In agreement with our identification of an amino-terminal region of p107 involved in kinase inhibition, we have shown here that pRB does not inhibit cyclin A-cdk2. Second, whereas pRB is not stably bound to this kinase before (10) or after (Fig. 4) phosphorylation, p107 remains bound to the kinase and inhibits its activity toward other substrates. Taken together, these experiments could provide an explanation for how pRB might function as a kinase substrate, while p107 and p130, with dedicated cyclin-binding domains, could function as specific inhibitors.

We have defined a second region of p107 and p130, spanning residues 67 to 69, that is required for efficient cyclin binding in addition to the LFG motif in the spacer region (43), and mutation of these amino acids results in the loss of inhibitory potential of the p107 amino-terminal domain. Furthermore, these p107 mutants were no longer bound by either cyclin-cdk2 complex in vivo, implying that binding of this portion of the molecule is important for both kinase and growth inhibition. Moreover, we showed that peptides corresponding to amino- and carboxy-terminal cyclin-binding sequences, but not a mutant version thereof, could specifically compete for the binding of cyclin A-cdk2 and cyclin E-cdk2 to p107 as well as restore histone H1 kinase activity inhibited by p107.

p107 displays an inhibitory spectrum similar to that of p21 and p27 with regard to the level of inhibition of cyclin A- and cyclin E-cdk2, although p107 may be more restricted than p21 and p27 in its specificity, since p107 does not appreciably inhibit cyclin D-cdk4 (data not shown). Interestingly, in our

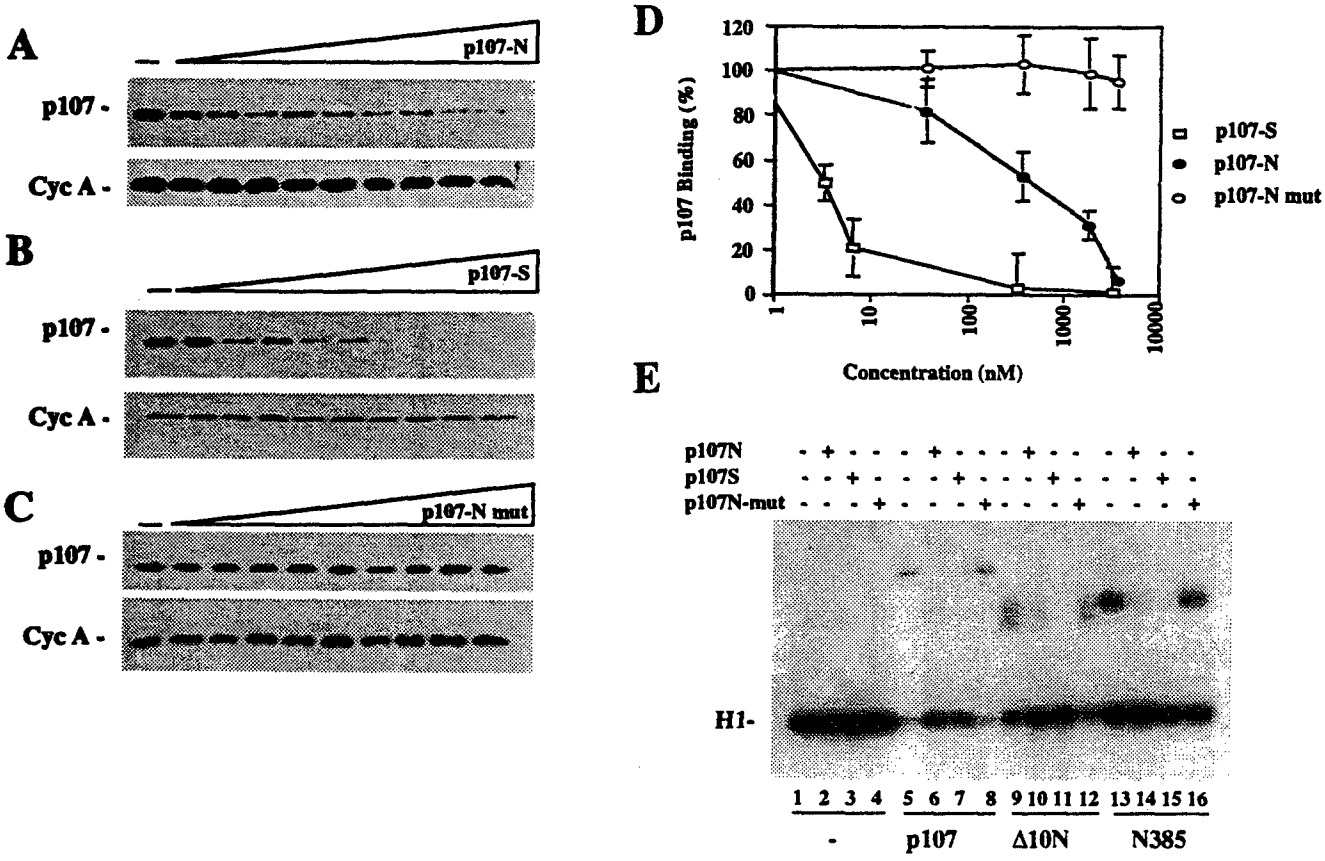


FIG. 10. Peptide binding competition of p107 to cyclin A (Cyc A)-cdk2. (A to C) Western blot analysis carried out after GST precipitation of GST-cyclin A-cdk2 shows the capacity of peptides p107S and p107N, but not p107N-mut, to compete the binding of p107 to cyclin A-cdk2. The concentrations of peptides range from 2 nM to 5 μM. The amount of cyclin A retained was not affected by addition of peptide and was included as a control. (D) Western blots were densitometrically scanned and quantified by utilizing NIH Image 1.61 software. The values indicate the means and standard errors of the means for three independent experiments. (E) Cyclin A-cdk2 kinase reaction. The peptides p107S and p107N, but not p107N-mut, were able to partially reverse the inhibition of cyclin A-cdk2 by p107 or Δ10N The peptides had no effect on the kinase reaction by themselves (compare lane 1 to lanes 2 to 4).

experiments, p107 inhibits cyclin E-cdk2 with a sixfold lower K_i than p21, suggesting that p107 may in some situations be a physiologically relevant inhibitor of cyclin E-cdk2. In addition to the ability to inhibit both cyclin A- and cyclin E-associated kinases, p107 and the CKI p21/WAF1 share another property, namely, the presence of dual cyclin-binding sites in their amino- and carboxy-terminal regions. It had been shown previously that p21/WAF1 utilizes two cyclin-binding domains, an amino-terminal one with an LFG motif known to be of critical importance in the binding of cyclin A to p27 (4, 17, 31, 33) and a second, related sequence near the carboxy terminus (Fig. 6A) (5).

Why is it necessary for p21 and p107 to engage cyclin-cdk2 complexes by using two binding sites? While we have not addressed the molecular architecture of cyclin-p107 or cyclin-p21 interactions here, we speculate that both cyclin-binding sites are essential for high-affinity interactions and potent inhibition. Indeed, although we could demonstrate detectable interactions between either binding site alone and cyclins in vitro, binding to full-length p107 was noticeably more efficient in vitro and in vivo, and both p107 binding sites were required in vivo to achieve efficient binding of cyclins and growth inhibition of C33A cells.

Although we have determined the existence of two cyclin-binding sites in p107, our studies point to a unique role for the amino-terminal region in kinase inhibition and growth suppression. The amino-terminal region of p107 is solely able to inhibit cyclin-cdk2 complexes in vitro, while equal or excessive amounts of the carboxy-terminal portion are unable to do so, although this portion is an efficient substrate for these kinases. A more detailed understanding of how these two regions of p107 differentially interact with cyclin-cdk2 will require a structural analysis of ternary p107-cyclin-cdk2 complexes.

It is intriguing to speculate that p107 and p130 may share overlapping functions as a CKI in the cell. p130 may share several properties of p107 both as an inhibitor and as a transcriptional regulator of E2F activity, as illustrated by numerous biochemical studies of these proteins and by recent mouse knockout experiments (6, 7, 23, 28, 32, 39). In the latter studies, it was shown that mice lacking not one but both proteins suffer lethal developmental abnormalities. Moreover, MEFs deficient in p107 and p130, but not either alone, showed strongly derepressed expression of specific genes, again suggesting that the two proteins have overlapping functions (24). Indeed, the two proteins also share a number of properties in our biochemical assays. For example, both appear to inhibit associated kinase activity (41), and both have sequences related to the LFG motif (8, 26). Thus, it is tempting to speculate that p130 may function in a manner analogous to p107. However, in our transfection assays, deletion of the amino-terminal cyclin-binding motif largely abolished the growth-suppressive activity of p107, while the growth arrest imposed by p130 was not altered as drastically by similar mutations (Fig. 6 and 8). This could suggest that p130 uses an additional mechanism(s) for restrain-

ing growth other than those used by p107. This possibility is currently under investigation.

The compensatory nature of p107 and p130 function has made it virtually impossible to study the normal role of each protein in vivo, and the function of different E2F complexes with p107 or p130 and cyclin-cdk2 remains obscure. In addition to studies related to kinase inhibition by these proteins, experiments are under way to dissect the transcriptional regulatory mechanisms through which each multiprotein complex functions in order to understand the normal role of p107 and p130 in cell cycle progression.

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